PHD

A study of prostacyclin production by the pregnant myometrium.

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Award date:
1983

Awarding institution:
University of Bath

A STUDY OF PROSTACYCLIN PRODUCTION

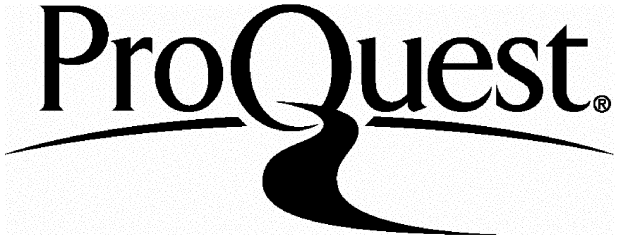
BY THE PREGNANT MYOMETRIUM.

submitted by M.P.Seed for the degree

of Ph.D. of the University of Bath

1983.

MSeed.



Twice two equals four: 'tis true,
But too empty, and too trite.
What I look for is a clue
To some matters not so light.

Busch (1909).

Acknowledgements

I would like to express my deepest gratitude to my supervisor Dr. K.I.Williams for his encouragement, scholastic and practical guidance throughout the last three years, and especially for allowing me the opportunity to undertake this research.

I am also indebted to the Head of School, Professor R.T.Parfitt and the Head of Pharmacology Group, Dr. P.H.Redfern for the use of the facilities of the School.

I owe great thanks especially to Mr. D.A.Bamford and the staff of the Princess Anne Wing of the Royal United Hospital for their patience and cooperation in obtaining samples.

I thank Mr. R.Francis and the staff of the Animal House for their good humoured assistance, Dr. R.V.Brunt, Department of Biochemistry, for his practical assistance and many helpful discussions and Mr. I.Middlemast, School of Electrical Engineering, for his help in "matters computing".

Finally I owe a great debt to the Wessex Area Health Authority and the University of Bath for funding the research, Dr. G.Blackwell (Wellcome Laboratories) for the gift of labelled dioleoyl-phosphatidylcholine and Wellcome Laboratories for the gift of prostacyclin.

TO MY MOTHER AND FATHER

SUMMARY

The rat and human pregnant myometrium and human placenta, when chopped and incubated in Tris-buffered saline (25%, 50% & 50% w/v respectively) released materials with anti-aggregatory activity. The myometrial activities were stable at pH 12.0, and unstable at pH 4.0. Optimum conditions for the release of the anti-aggregatory activities were 15, 30 and 15 minutes incubation respectively at 20°C at pH 8.0. The release of anti-aggregatory activity by human myometrium was stimulated by arachidonic acid and phospholipase A_2 (PLA_2) and inhibited by indomethacin. Human myometrial incubate did not possess adenosine-diphosphatase activity. This anti-aggregatory activity released by pregnant rat and human myometria was attributed to prostacyclin (PGI_2). Human placental anti-aggregatory activity was unstable at both pH 12.0 and pH 4.0, was not inhibited by administration of indomethacin and had the ability to degrade adenosine diphosphate.

PGI_2 synthesis by human pregnant myometrium was low up to week 37 of pregnancy and then increased progressively to a maximum at week 40 (term). Two patients who did not progress to labour by week 42 had a low capacity to release PGI_2. Incubation of human myometrial tissue with oxytocin and ergometrine did not alter PGI_2 synthesis. PGI_2 inhibited the spontaneous contractility of human pregnant myometrium bathed in Krebs' solution in 3/4 samples and reduced basal tone in 2/4.

Studies on pregnant rat myometrial PGI_2 synthesis showed that removal of the deciduum by scraping with a microscope slide resulted in greatly increased synthesisof PGI_2 compared to teasing. Chopping increased the rate of synthesis of the scraped samples, and maximal synthesis of the teased samples.

Pre-incubation of scraped myometrial tissue with uterine stimulant drugs (oxytocin, angiotensin & 5-hydroxytryptamine [5-HT]) stimulated PGI_2 synthesis in a dose related fashion. Methysergide, whilst reducing synthesis, inhibited the stimulant effect of 5-HT. The uterine relaxant salbutamol inhibited synthesis by day 21 but not day 19 pregnant myometerium, and was antagonised by propranolol. The adenylate cyclase stimulant, forskolin, reduced PGI_2 synthesis.

Rat uterine homogenate, when incubated in HEPES buffer (pH 8.0; + Triton X-100) released ^{3}H-oleic acid from 1'-oleoyl, 2'-[^{3}H]-oleoyl-phosphatidylcholine (^{3}H-dPC). Two methods were assessed for the separation of released oleic acid from the ^{3}H-dPC. Thin layer chromatography was found to be the method of choice.

The release of ^{3}H-oleic acid by late pregnant rat uterine homogenate acid was Ca^{++} dependent, protein dependent, had a pH optimum at pH 8.0 and was inhibited by mepacrine and tetracaine. This activity was attributed to PLA_2. Perfusion of the uterine vascular bed with Krebs' solution to remove blood elements increased activity as measured by this method.

CONTENTS

1.0) Introduction

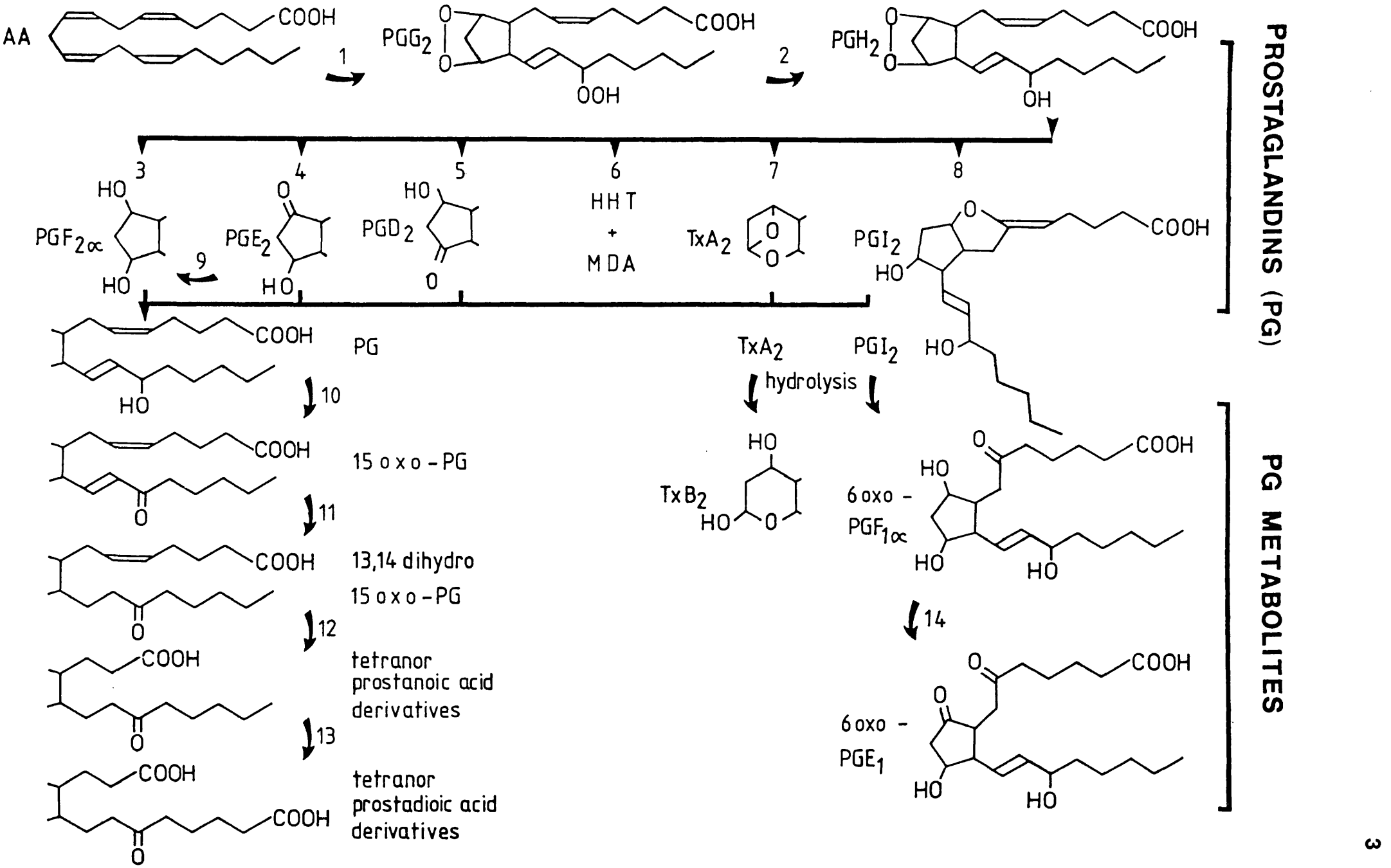
1.1) HISTORY

The initial discovery of a principle possessing vasodepressor and smooth muscle stimulant activity in semen is attributed to Goldblatt (1933) and von Euler (1937) which was subsequently named prostaglandin (PG). von Euler characterised this as a lipid soluble fatty acid which contained an hydroxy group.Bergstrom and Sjoval (1957;1960a,b) succeeded in isolating and determining the structure of two pure PGs from seminal fluid and called them PGF and PGE denoting preferential solubility in phosphate buffer and ether respectively. Further prostaglandins were designated by letters from the beginning of the alphabet and have now reached the 'I' series.

The structure and configuration of $PGF_{2\alpha}$ was confirmed by X-ray crystallography (Abrahamsson, Bergstrom, & Samuelsson, 1962), as a member of the class of compounds chemically related to prostanoic acid. Research then ascertained the biosynthetic pathway for the PGs and was first achieved in 1964 (van Dorp, Beerthuis, Nugteren & Vonkeman 1964a). Bergstrom simultaneously showed that arachidonic acid (AA) was converted to PGE_2 by sheep vesicular glands which was subsequently found to be secondary to the production of endoperoxide intermediates (Hamberg & Samuelsson 1973; Nugteren & Hazelhof 1973). These are sythesised from AA and converted to $PGF_{2\alpha}$, PGD_2, and PGE_2 as well as a non-prostanoid group of compounds called thromboxanes (Hamberg, Svensson & Samuelsson 1976). Under a year later 6-oxo-$PGF_{1\alpha}$ was islolated from rat stomach, guinea-pig lung and rat carageenan-induced granuloma (Pace-Asciak 1976; Chang, Murota, Matsuo & Tsurufuji, 1976; Dawson, Boot, Cockerill, Mallen & Osborne, 1976). Attention was then drawn to a report that PG endoperoxides were converted, by arterial walls, to a potent anti-aggregatory substance, PGX (Gryglewski, Bunting, Moncada, Flower & Vane,

Figure 1) Structure and pathway for arachidonic acid metabolism via the cyclooxygenase pathway.

Prostaglandin (PG) synthesis.	Drug stimulation(+) or inhibition (-).	
1) Cyclooxygenase	Aspirin	-
	Indomethacin	-
2) Peroxidase	Tryptophan	+
	Adrenaline	+
3) PG-endoperoxide F-reductase		
4) PGE-isomerase		
5) PGD-isomerase	Albumin	+
6/7) Thromboxane synthetase	1n-butyl-imidazole	-
	Nictindole	-
8) Prostacyclin synthetase	15-HPAA	-
9) 9 Keto-reductase	Bradykinin	+
Prostaglandin (PG) metabolism		
10) PG 15-dehydrogenase	Aspirin	-
	Indomethacin	-
	PGB	-
11) PG 13-reductase		
12) -oxidation, 2 steps.		
13) -oxidation		
14) PG 9-dehydrogenase.		


AA
COOH
1
PGG2
OOH
2
PGH2
OH
3
4
5
6
7
8
HO
PGF2∝
HO
9
O
PGE2
HO
HO
PGD2
O
HHT
+
MDA
TxA2
O
O
PGI2
HO
COOH
PG
10
15 oxo - PG
11
13,14 dihydro
15 oxo - PG
12
tetranor
prostanoic acid
derivatives
13
tetranor
prostadioic acid
derivatives
TxA2
hydrolysis
PGI2
HO
TxB2
HO
HO
O
6 oxo -
PGF1∝
HO
HO
14
6 oxo -
PGE1
O
HO
HO
PROSTAGLANDINS (PG)
PG METABOLITES

1976). PGX was later renamed as prostacyclin (PGI_2) and 6-oxo-$PGF_{1\alpha}$ ascertained to be the hydrolysis product of PGI_2 in acid or aqueous media.

1.2) CHEMISTRY

1.2 i) Chemistry of prostaglandins.

PGs are C-9 oxygenated chemical derivatives of prostanoic acid, a twenty carbon monocarboxylic acid containing a cyclopentane ring joining the two carbons at C-8 and C-12 numbered from the carboxyl group. The PGs are labelled alphabetically and are mainly distinguished by the type and orientation of the substituent groups in the ring. PGA , PGB and PGC contain a C-9 ketone and differ by the position of a double bond within the cyclopentane ring, whilst PGD, PGE and PGF are distinguished by the presence of hydroxyl and/or ketone groups at C-9 and C-11. PGG and PGH are cyclic 9,11 endoperoxides, and PGG (and its metabolites) is exceptional in containing a hydroperoxy group at C-15 instead of the hydroxyl group found with all other PGs (see figure 1 for structures).

These groups are further subdivided into the 1, 2, or 3 series according to the number of double bonds found in the side chains. These are derived from 8,11 eicosadienoic acids. Cyclo-oxygenase will thus convert 8,11,14 eicosatrienoic acid (dihomoγlinoleic acid, DHγLA) to the '1' series, 5,8,11,14 eicosatetraenoic acid (arachidonic acid, AA) to the '2' series and 5,8,11,14,17 eicosapentaenoic acid to PGs of the '3' series. The configuration of the double bonds are trans, cis , trans respectively. The isomeric form of the F-series is further denoted by subscripts which indicate the orientation of the C-9 hydroxyl group as above (α) or below (β) the plane of the cyclopentane ring.

The prostacyclins (PGI) and thromboxanes are non-prostanoid transformation products of the endoperoxide intermediate. PGI is bicyclic containing the cyclopentane ring and a furan ring between C-6 and C-9 of the prostanoic acid molecule. The thromboxanes contain a pyran ring as opposed to the cyclopentane ring and are divided into two series. The oxetone formation of thromboxane A (TxA) is rapidly hydrolysed to form thromoxane B (TxB). PGI, TxA and TxB are split into the mono, bis or tris series as with the classical PGs and derived from the same precursors.

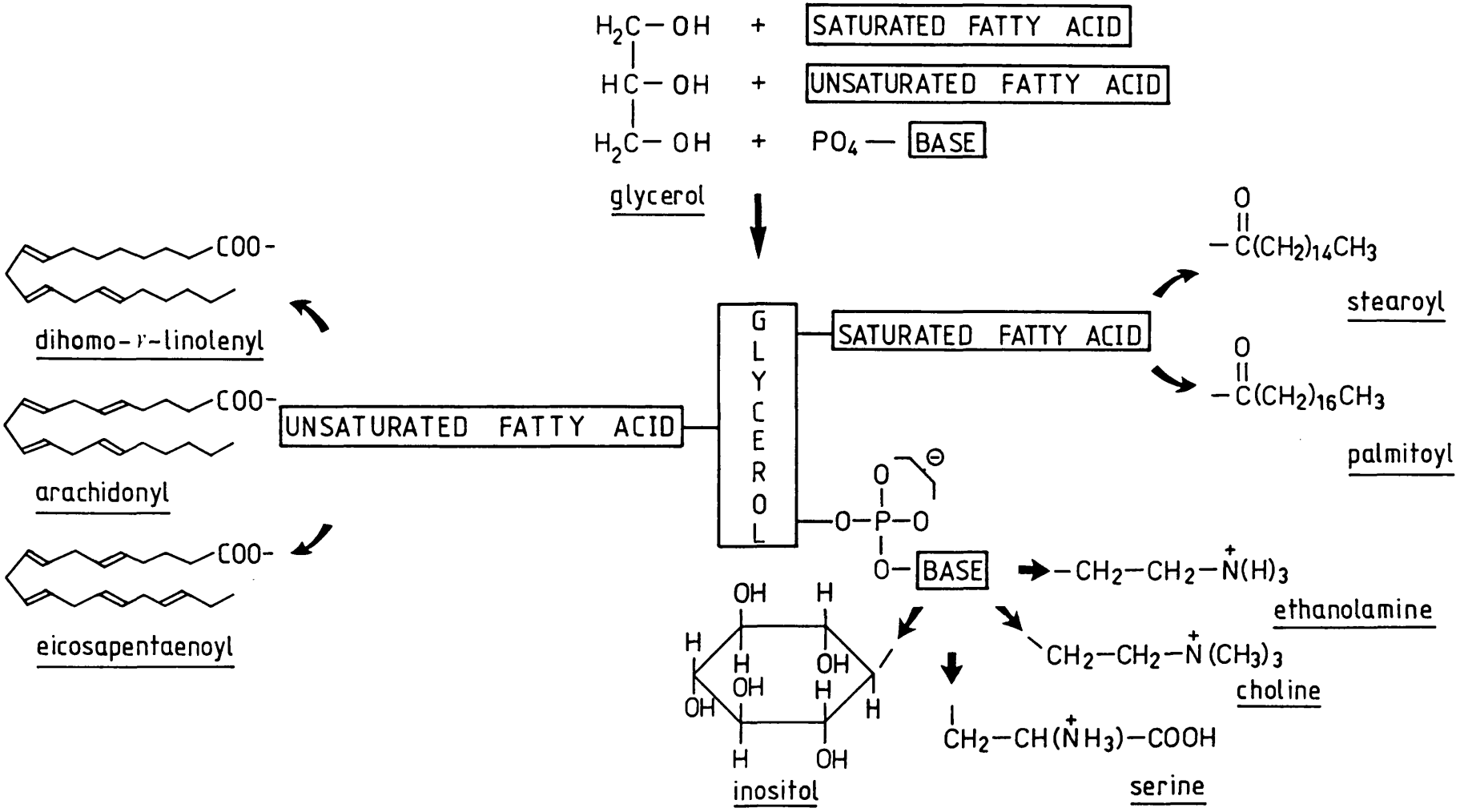
1.2 ii) Chemistry of phospholipids

The phospholipids (also named phosphatides) relevant to PG biochemistry are derivatives of glycerol which can be regarded as the chemical backbone to which the other components are linked (see figure 2). The 1'(α) position is generaly occupied by the saturated fatty acids stearic or palmitic acids whilst the 2' (β) position (which is assymetric) is occupied by the PG-precursor unsaturated fatty acids : DH LA, AA or 5,8,11,14,17-eicosatetraenoic acid. Serine, inositol, choline and ethanolamine are the most common bases phosphorylated at position 3 (γ) . Thus a phospholipid containing a 1' stearic acid, 2' AA and 3' inositol would be called 1'-stearoyl,2'-arachidonoyl-phosphatidylinositol. This is more often shortened to phosphatidyl-inositol and the saturated and unsaturated fatty acids not specifically noted.

1.3) Arachidonic acid release and metabolism

AA is concentrated as the ester mainly in the form of glycero-phospholipids and is the preferred unsaturated fatty acid (Danon,

Figure 2) The structure of the commonly occurring glycero-phospholipids into which prostaglandin precursors are incorporated (illustrating the glycerol backbone).


H2C—OH + SATURATED FATTY ACID
HC—OH + UNSATURATED FATTY ACID
H2C—OH + PO4 — BASE
glycerol
GLYCEROL
SATURATED FATTY ACID
UNSATURATED FATTY ACID
—C(CH2)14CH3
stearoyl
—C(CH2)16CH3
palmitoyl
dihomo-γ-linolenyl
arachidonyl
eicosapentaenoyl
COO-
O—P—O
O— BASE
—CH2—CH2—N(H)3
ethanolamine
CH2—CH2—N(CH3)3
choline
CH2—CH(NH3)—COOH
serine
inositol

Heimberg & Oates, 1975) even though DHYLA is an intermediate for AA synthesis in the rat (Marcel, Christiansen & Holman, 1968). AA can be obtained in the diet or synthesised from dietary linolenic acid by desaturation and chain elongation in the liver forming DHYLA. This is further desaturated to form a Δ^{5-6} double bond characteristic of the 2-series of PGs. AA is metabolised through two independant pathways: prostaglandin synthetase and lipoxygenase to form the prostaglandins and hydroxyacids respectively.

1.3 i) Role of phospholipases in AA release

Phospholipases are a large group of enzymes that hydrolyse phospholipids. They differ in their specificity, pH optima, subcellular localisation and requirements for calcium ions. Those required for PG biosynthesis are phospholipases of the A_1, A_2, C and D series (see figure 3). Phospholipase D is only found in plants and will not be discussed further. PLA_1 hydrolyses the saturated fatty acid from the 1' position of the glycerophospholipid leaving a 1'lysophosphatide whilst PLA_2 is specific for the 2' position releasing the unsaturated fatty acid and leaving the 2'-lysophosphatide. PLC releases the phosphorylated base from position 3' leaving 1,2 diacylglycerol. The lysophosphatides are cytotoxic and are reacylated to phosphatides or further degraded by lysophosphatidases.

PGs are not stored within cells (Piper & Vane,1971) and are synthesised de novo prior to release. The free acids are required by cyclo-oxygenase (Lands & Samuelsson,1968; Vonkeman & van Dorp,1968) and free acid levels are low (Kunze & Vogt,1971). Kunze and Vogt also suggested that the release of free AA may be controlled by a PLA which

Figure 3) A diagram to illustrate the actions of different phospholipases on the phospholipid 1-stearoyl,2-arachidonoyl-phosphatidyl-inositol.

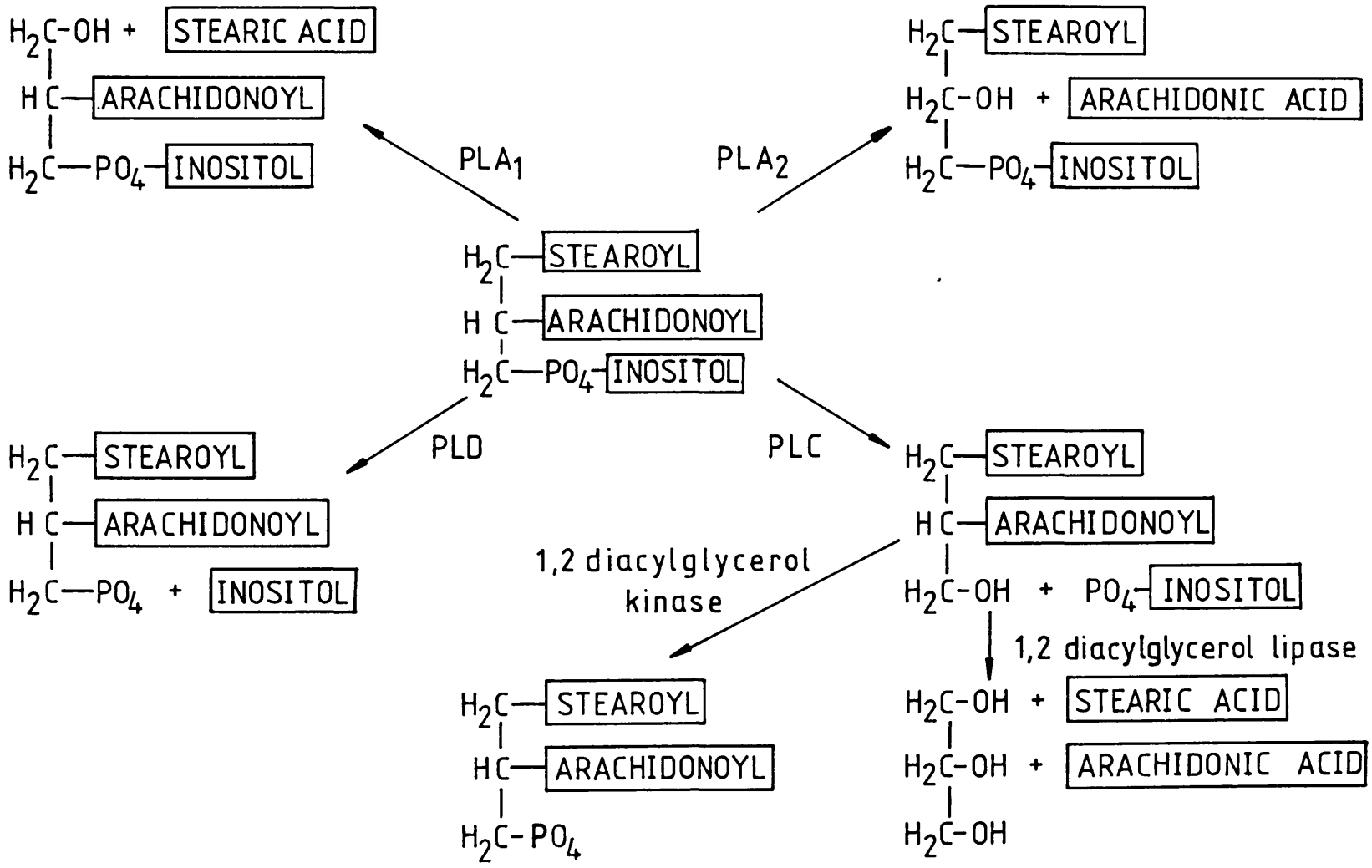
PLA_1 - Phospholipase-A_1

PLA_2 - Phospholipase-A_2

PLC - Phospholipase-C

PLD - Phospholipase-D

The 1,2-diacyl-glycerol produced under the action of phospholipase-C is further altered by 1,2-diacylglycerol kinase and 1,2-diacylglycerol lipase to release phosphatidic acid and glycerol respectively.


H2C-OH + STEARIC ACID
HC—ARACHIDONOYL
H2C—PO4—INOSITOL
PLA1
PLA2
H2C—STEAROYL
H2C-OH + ARACHIDONIC ACID
H2C—PO4—INOSITOL
H2C—STEAROYL
HC—ARACHIDONOYL
H2C—PO4—INOSITOL
PLD
PLC
H2C—STEAROYL
HC—ARACHIDONOYL
H2C—PO4 + INOSITOL
1,2 diacylglycerol kinase
H2C—STEAROYL
HC—ARACHIDONOYL
H2C-OH + PO4—INOSITOL
1,2 diacylglycerol lipase
H2C—STEAROYL
HC—ARACHIDONOYL
H2C-PO4
H2C-OH + STEARIC ACID
H2C-OH + ARACHIDONIC ACID
H2C-OH

may be the rate limiting step in PG biosynthesis. AA and PLA_2 administration leads to a large increase in PG release when infused through frog intestine (Bartels, Kunze, Vogt & Willis,1970). Bradykinin-induced PG release from guinea-pig lung is inhibited by mepacrine, a PLA_2 inhibitor, whilst AA stimulated release is not (Vargaftig & Dao Hai,1972). Radiolabelled AA is incorporated into the neutral and 2'lecithin-like phospholipid pools of the guinea-pig spleen and radiolabelled AA is released on shaking from the phospholipid fraction, not the neutral lipids (Flower & Blackwell,1976). These results show that PGs are not released themselves by cleavage from the phospholipids but that the precursor AA is.

More recently it has been found that platelet aggregation results in a loss of AA from the phosphatidylinositol (PI), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) pools in the plasma membranes (Blackwell, Duncombe, Flower, Parsons & Vane,1977). This was taken as evidence that platelet AA is released by PLA_2. However Rittenhouse-Simmonds (1979) and Bell, Kennerly, Stanford & Majereus (1979) have provided evidence for a PI specific PLC which releases the corresponding 1,2-dialcylglycerol which is then subsequently metabolised. 1,2-diacyl-glyceryl lipase (diglyceride lipase) cleaves both the unsaturated and saturated fatty acids from the diglyceride making it difficult to distinguish this action from PLA_2. This does not preclude a role for platelet PLA_2 since AA is still released from PC and PE which are not substrates for the PI specific PLC.

1.3 ii) Prostaglandin and thromboxane synthesis

Figure 1 illustrates the mechanism of PG biosynthesis. AA is initially dioxygenated to PGG_2, involving C-11 and C-15 hydro-

peroxidation, C-13 dehydrogenation, with cyclisation at C-8 and C-12 by PG endoperoxide synthetase (Miyamoto, Ogino, Yamamoto & Hayaishi,1976). A peroxidase catalyses the C-15 hydroperoxide to the hydroxy group of PGH_2 (van Dorp, Buytenhek, Christ-Hazelhof, Nugteren & van der Oudera,1978).

PG endoperoxide F-reductase is present in the cytosol (van Dorp et.al., 1978) converting PGH_2 to $PGF_{2\alpha}$. PGE-isomerase utilises PGG_2 (but prefers PGH_2) yielding 15-hydroperoxy-PGE_2 (and PGE_2) (Ogino, Miyamoto, Yamamoto & Hayaishi,1977).

Controlled experiments have shown that PGE is non-enzymatically dehydrated to PGA when the pH is allowed to drop below 3.5 or high temperatures occur prior to extraction (Jonsson & Powers,1981). The enzymatic production of PGD_2 has also been questioned, as has its role in biological systems, since it is easily formed non-enzymatically (like PGE_2) from PGH_2 (Hamberg & Samuelsson,1974a; Nugteren & Hazelhof,1973). However rat spleen (Christ-Hazelhof & Nugteren,1979) and rat brain (Shimizu, Yamamoto & Hayaishi,1979) have yielded PGD-isomerases.

Thromboxane synthetase was first discovered in platelet microsomes by Needleman, Moncada, Bunting, Vane, Hamberg & Samuelsson (1976) after thromboxane was found to be present in platelet extracts (Hamberg & Samuelsson,1974b; Hamberg, Svensson & Samuelsson,1975). Equal amounts of 12-hydroxy-heptadecatrienoic acid with malondialdehyde (Diczfalusy, Falardeau & Hammarstrom,1977) are released with TxA_2.

Prostacyclin synthetase was originally described in arterial microsomes (Moncada, Gryglewski, Bunting & Vane,1976). The potent anti-aggregatory factor released was named PGX (Gryglewski et.al., 1976) and found to be an arterial relaxant (Moncada et. al.,1976). PGX was renamed

PGI_2 (Johnson, Morton, Kinner, Gorman, McGuire, Sun, Whittaker, Bunting, Salmon, Moncada & Vane,1976).

Finally 9-oxo reductase activity, converting PGE to PGF is found in blood, heart and liver (Hensby,1975). It has also been noted that the opposite reaction can occur(i.e. dehydration converting PGF to PGE) (Pace-Asciak & Miller,1974) in the rat kidney.

1.3 iv) Lipoxygenase pathway

A further transformation of AA to hydroxy fatty acids can occur via the lipoxygenase pathway to form a newly discovered group of compounds named leukotrienes (Samuelsson & Hammarstrom,1980). These were first found to be released from leucocytes (Borgeat & Samuelsson,1979) and their structure elucidated as hydroxy or hydroperoxy-eicosapolyenoic acid derivatives depending apon the precursor fatty acid as with the PGs. The lipoxygenase products also include tri, di or mono-hydroxyeicosapolyenoic acids. The leukotrienes are potent constrictors of airways (Lewis, Austen, Drazen, Clark, Marfat & Corey,1980) and increase vascular premeability. Leukotriene B_4 is chemotactic and chemokinetic and the hydroxy acid 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE) inhibits PGI_2 synthetase (Seigel, McConnel, Abrahams, Porter & Cuatrecasas,1979) .

1.3 v) Prostaglandin and thromboxane metabolism

The rapid loss of biological activity of the PGs and thromboxanes are both a result of enzymic oxidation by prostaglandin-15-dehydrogenase (PGDH) (Samuelsson, Granstrom, Green, Hamberg & Hammarstrom,1975) and the hydrolysis of PGI_2 and TxA_2 to 6-oxo-$PGF_{1\alpha}$ and TxB_2 respectively (see figure 1). The 15-oxo metabolites may then be reduced by Δ^{13}-

reductase to form 15-oxo-13,14-dihydroPGs (Samuelsson et. al.,1975). Further chain shortening by β-oxidation leads to dinor- or tetranor-compounds (Hamberg,1968) and ω-oxidation leading to dioic acids. PGF and PGE were the first PGs described to undergo metabolism by this pathway (Samuelsson et. al.,1975). PGD_2 also undergoes metabolism by these pathways but may also form dinor-$PGF_{1\alpha}$ metabolites (Ellis, Smigel, Oates, Oelz & Sweetman,1979). Thromboxane release from sensitised guinea-pig lung illustrates that TxA_2 is a substrate for PGDH as 15-oxo-dihydroTxB_2 is the major metabolite synthesised (Boot, Cockerill, Dawson, Mallen & Osborne,1978) and not dinorTxB_2 <u>in vivo</u> (Svensson,1979). TxB_2 is not a substrate for PGDH <u>in vitro</u> and is not metabolised to 15-oxo-dihydroTxB_2 in the monkey (Kindahl,1977). TxA_2 hydrolysis may then be preceded by metabolism by PGDH and Δ^{13}-reductase since TxA_2 is rapidly trapped by albumin which increases the plasma half life (Folco, Granstrom & Kindahl, 1977). TxB_2 is only produced on acidification of plasma proteins on extraction of TxA_2 (Maclouf, Kindahl, Granstrom & Samuelsson,1980).

PGI_2 also undergoes hydrolysis to 6-oxo-$PGF_{1\alpha}$, however only β and ω-oxidation products of the 6-oxo-$PGF_{1\alpha}$ are found on administration of 6-oxo-PGF_1~ to rats (Pace-Asciak, Carrera & Domazet,1977). 6-oxo-$PGF_{1\alpha}$ is a poor substrate for PGDH but other metabolites of PGI_2 have been found (McGuire & Sun,1978). These were metabolised in the normal way by C-15 dehydrogenation and Δ^{13}-reduction. There is also the possibility of spontaneous hydrolysis of PGI_2 to 6-oxo-$PGF_{1\alpha}$ and then C-9 dehydrogenation to yield the anti-aggregatory product 6-oxo-PGE_1 (Wong, Malik, Sun, Lee & McGiff,1978).

1.3 vi) Pharmacological manipulation of the arachidonic acid pathway

Phospholipases A_2 and C are both Ca^{++} dependant thus calcium ionophores should activate the AA cascade. This is true for PLA_2 (Pickett ,Jesse & Cohen,1977) but Ca^{++} mobilisation by A23187 does not activate PLC in intact cells (Lapetina & Cuatrecasas,1979). Mepacrine is a direct inhibitor of PLA_2 (Vargaftig & Daotti,1972) as is tetracaine (Kunze, Bohn & Vogt, 1974) and p-bromophenacylbromide (Mitchell, Poyser, & Wilson,1977). Steroidal ani-inflammatory drugs inhibit PLA_2 (Flower,1978) probably via the de novo synthesis of an endogenous PLA_2 inhibitor (Flower & Blackwell,1979). Inhibition of PLA_2 will limit the production of all AA metabolites.

Selective inhibition of AA metabolism via the PG synthetase pathway using aspirin was first reported by Vane (1971). Cyclo-oxygenase was at one time thought to be inseparable from peroxidase, however it is now known that aspirin specifically inhibits cycloogenase (van Dorp et. al.,1978). Indomethacin, a reversible cyclo-oxygenase inhibitor (Crook & Collins,1977) either reduces enzyme affinity for the substrate (Lands, La Tellier, Rome & Vanderhoek,1973) or acts by competitive inhibition (Flower, Cheung & Cushman,1973). Aspirin acetylates cyclo-oxygenase and thus its action is irreversible (Roth, Stanford & Majerus,1975).

Peroxidase on the other hand is stimulated by several hydro-peroxides (Okhi, Ogino, Yamamoto & Hayaishi,1979), tryptophan (Miyamoto et. al.,1976) and adrenaline (Sih, Takeguchi & Foss,1970).

Of the isomerases only PGE-isomerase has been successfully inhibited specifically, by 5,8,11-eicosatrienoic acid (van Evert, Nugteren & Van Dorp,1978). On the other hand several inhibitors of the synthetase enzymes for PGI_2 and TxA_2 have been elucidated. 15-hydro-peroxyarachidonic acid (Moncada, Gryglewski, Bunting & Vane,1976) and

other lipid hydroperoxides (Salmon, Smith, Flower, Moncada & Vane,1976) inhibit PGI_2 synthetase. Tranylcypromine (Gryglewski et.al., 1976), nicotine (Wennmalm,1978) and 3-hydroperoxy-3-methyl-2-phenyl-3-indole (Terashita, Nishikawa, Terao, Nakagawa & Hino,1979) are also inhibitors. However the action of tranylcypromine is now consisered to be non-selective (Rajtor & de Gaetano, 1979).

Thromboxane synthetase is inhibited by imidazole analogues (Moncada, Bunting, Mullane, Thorogood, Vane, Raz & Needleman,1977). 1-n-butylimidazole has no phosphodiesterase inhibitory activity and is thus more useful as an inhibitor of thromboxane synthetase than imidazole (Blackwell, Flower, Russell-Smith, Salmon, Thorogood & Vane,1978) since TxA_2 is aggregatory (Hamberg, Svensson & Samuelsson, 1975) and elevated cAMP levels are anti-aggregatory in platelets (Mills & Smith, 1971). Lack of selectivity and of oral activity have precluded the use of inhibitors such as benzydamine, (Diczfalusy & Hammarstrom,1977), nictindole (Gryglewski, Korbut, Ocetkiewicz & Stachura,1978) and N-0164 (Eakins, Rajadhyaksha & Schroer,1976).

Lipoxygenase has proved equally difficult to inhibit selectively. Dual cyclooxegenase-lipoxygenase inhibitors have been useful since indomethacin is relatively inactive against lipoxygenase and thus the pharmacological or physiologiacal effects of the two enzymes or their products can be differentiated by comparison. BW-755C, a pyrazolidine derivative, inhibits PGE_2 synthesis by carageenan sponges in rats as well as platelet lipoxygenase (Higgs, Flower & Vane,1979; Higgs, Eakins, Mugridge, Moncada & Vane,1980). Eicosa-5,8,11,14-tetraynoic acid (TYA) is the other most commonly used dual inhibitor (Hamberg & Samuelsson,1974) with similar IC_{50} values for both enzymes.

The metabolic degradation of the PGs can also be manipulated by the addition of NAD^+ and other NAD analogues as PGDH is an NAD^+ dependant enzyme. PG analogues such as the isomer of PGE_1, 15R-PGE_1 and PGs of the B series inhibit PGDH (Nalcano, Angaard, & Samuelsson, 1969). Aspirin and indomethacin have also been reported to inhibit bovine lung PGDH (Hansen, 1974) but the concentrations needed are considerably higher than those required to inhibit cyclo-oxygenase.

1.4) Arachidonic acid metabolism and uterine function

Smooth muscle contracting substances present in human menstrual fluid were first reported in 1957 by Pickles and characterised as PGs (Eglington, Raphael, Smith, Hall & Pickles,1963). PGs have since been identified in the uterine tissues of various species (for reviews see Williams & Vane,1975; Horton & Poyser,1976)

Studies of PG production of human and rat uteri show great variations in AA metabolism in the 2 uterine fractions constituting the uterus. Human endometrial PGE and PGF levels are high as compared to 6-oxo-$PGF_{1\alpha}$ which is undetectable by GCMS (Kelly, Smith, Cooper & Abel,1980). This is confirmed using human endometrial homogenates incubated with ^{14}C-AA (Abel & Kelly,1979). The human myometrium was also shown to synthesise large amounts of 6-oxo-$PGF_{1\alpha}$ compared to PGE and PGF and this was increased considerably on incubation with endometrium. The human pregnant myometrium and deciduum has however also been reported to synthesise equal amounts of PGE_2 and $PGF_{2\alpha}$ (Sykes, Williams & Rogers,1975). But cofactors were used and it has since been found that the human decidua and amnion release considerably greater quantities of PGE and PGF than the myometrium in vitro without cofactors (Satoh, Yasumizu, Kawai, Ozaki, Wu, Kinoshita & Sakamoto,1981). The myometrium

releases ten times more 6-oxo-$PGF_{1\alpha}$ than decidua and amnion. Similar results have been found with the rat pregnant uterine fractions. The decidual microsomes synthesise PGE_2, $PGF_{2\alpha}$ and TxB_2 (Williams & Downing,1977) and the myometrium mainly 6-oxo-$PGF_{1\alpha}$ (Downing & Williams,1977). It has also been confirmed that chopped late pregnant rat myometrium releases more PGI_2-like anti-aggregatory material than the decidua (Williams, Dembinska-Kiec, Zmuda & Gryglewski,1978) but that this difference is due to the presence of blood elements in the decidual tissue releasing a lipoxygenase product which inhibits myometrial PGI_2 synthesis (ElTahir & Williams, 1981). Human myometrial tissue also releases PGI_2-like anti-aggregatory activity (Omini, Folco ,Pasargiclion, Fano & Berti,1978) and this increases at term (Bamford, Jogee & Williams,1980) in a manner similar to that found in the rat (Williams & ElTahir, 1980a).

PG catabolism within the pregnant uterus has been mainly investigated using human tissues. The placenta is certainly a prime site for PGDH activity (Jarabak,1972)). The foetal membranes also contain significant quantities of PGDH as well as Δ^{13}-reductase (Keirse & Turnbull,1975 ; Keirse, Williamson & Turnbull,1975). PGDH activity within the myometrium and decidua is very low and it seems that tissues of trophoblastic origin (placenta, chorion and amnion) contain the most significant amounts of PGDH.

1.5) Role of prostaglandins in parturition

Evidence for the role of PGs in rat and human parturition has necessarily come from radically different sources.

Karim and Devlin (1967) first showed that PGF could be found in

amniotic fluid of women during labour. The PGF was then shown to be greater at term than mid-pregnancy (Keirse, Flint & Turnbull,1974) and plasma levels of 15-oxo-$PGF_{2\alpha}$ have been shown to rise during labour (Karim ,1968). Keirse, Mitchell and Turnbull (1977) found that pregnant women who had low levels of PGF_2 in their amniotic fluid did not progress adequately to the first stage of labour in contrast to those with high PGF levels (however care must be taken in interpreting these results since frequent amniotomy or amniocentesis may induce PG release [Mitchell, Keirse, Anderson & Turnbull,1977]).

Primary evidence of PG involvement in the rat parturition process is that PGF_2 release from rat pregnant uterus *in vitro* increases from day 17 to 22 and decreases post-partum (Williams,1973; Williams,Sneddon & Harney,1974). This preceded the discovery that PGF levels increase coincidentaly with oestradiol in the ovarian venous plasma of rats, then drops on day 23, the day after delivery (Labhsetwar & Watson,1974). PGF release *in vitro* correlates well with spontaneous uterine contractions and both are inhibited by indomethacin (Williams,1973). PGs are not released as a consequence of uterine contractility as papaverine inhibits pregnant uterine activity without affecting PGF release (Aiken,1974).

The low release of PG on day 17 in the rat is due to the lack of available AA within the rat uterus as addition of AA and PLA_2 *in vitro* elicits uterine contractions with a release of PGF (Parnham, Sneddon & Williams,1975). Although this experiment has not been reported using human myometrium *in vitro*, free amniotic AA and PGF levels are markedly raised in women in labour (MacDonald, Shultz, Duenhoelter, Gant, Jimenez, Pritchard, Porter & Johnston,1974). Human foetal membranes are enriched in AA (Schwartz, Schultz, MacDonald & Johnston,1975) found in the 2' position of the glycerophospholipids(Okita, MacDonald &

Johnston,1980) which is cleaved by PLA_2 (Okazaki, Okita, MacDonald & Johnston,1978). The AA content may be reduced during labour (Schwartz et. al.,1975) but Filshie and Anstey (1978) found no change in free and esterified AA in tissues obtained before and during labour in women . It must be noted here that placental PGDH does not change before or during spontaneous or induced labour (Keirse, Hansenns, Hicks & Turnbull,1976; Keirse, Hicks & Turnbull,1976) although similar changes could be observed using similar methods in sheep (Keirse, Mitchell & Flint,1977). Thus a reduction in PG catabolism cannot account for the increased PG release at term in women. PGE and PGF are also synthesised by foetal membranes (Keirse & Turnbull,1976).

PGE and PGF are both powerful oxytocic agents in the pregnant uteri of humans (Embre,1969; Wiqvist, Bygdemann & Kirton,1971) and rats (Fuchs & Mok,1973) and thus may facilitate the expulsion of the foetus at term. PG administration in women (Karim,1971) and rats (Buckle & Nathanielz,1973) induces labour and abortion. Whether this is due to the oxytocic action of PGs or not was questioned when Horton and Poyser (1976) found that $PGF_{2\alpha}$ decreased serum progesterone levels in various species, probably secondarily to luteolysis thus relieving progesterone block leading to an increase in uterine sensitivity to other stimuli (Csapo,1969). Further support for this hypothesis comes from the observation that preadministration of progesterone attenuates the effect of $PGF_{2\alpha}$ in the rat (Gutnecht, Cornette & Pharris, 1969; Fuchs & Mok,1973).

Abortion induced by amniotic instillation of saline , however, stimulates the release of PGF into the amniotic fluid of women (Gustavii & Green,1972) and the time to abortion is prolonged with the

administration of indomethacin and aspirin before induction (Waltman et. al.,1972; 1973a). Aspirin also increases gestational length in women (Lewis & Schulman,1973) and rats (Chester, Dukes, Slater & Walpole, 1972).

PGI_2 is oxytocic in the non-pregnant (Omini, Moncada and Vane, 1977) and pregnant rat (Williams, ElTahir & Marcinciewicz,1979) uteri in vitro but in concentrations far higher than PGE_2 or PGF . Subthreshold concentrations also sensitizes the pregnant rat uterus to cause a 3-fold potentiation of oxytocin. However PGI_2 is relaxant when administered in vitro to the human pregnant uterus (Omini et. al.,1979) and antagonises the effect of other PGs in the non-pregnant human myometrium (Sanger & Bennet,1980). These results have been contradicted by Wiqvist and his group who showed an oxytocic action of PGI_2 on human non-pregnant myometrium and uterotubal junction (Wiqvist, Lindblom & Willhelmsson,1979).

1.6) Oxytocic and tocolytic drugs and uterine prostaglandin synthesis

The possibility that oxytocic drugs, including oxytocin, may act by stimulating uterine PG synthesis has been questioned. Indomethacin antagonises the effect of oxytocin on the in vitro non-pregnant rat uterus but failed to do so completely in 8/10 pregnant uteri (Vane & Williams,1973). Indomethacin and meclofenamate inhibit the effect of bradykinin and 5-hydroxytryptamine (5-HT) (Sorrentino, Cepasso & DiRosa,1972) and angiotensin II (Baudouin-Legros, Meyer & Worcel,1974). Indomethacin also delays oxytocin induced parturition in rats (Fuchs, Smitasiri & Chantharaksri,1976). Oxytocin in women is ineffective at inducing labour or uterine contractions before week 20 , after which there is an increase in sensitivity (Anderson & Turnbull, 1968). Also of

great interest is the finding that oxytocin is not effective for induction of labour in women with hydatidiform mole, intrauterine death or serious foetal abnormalities - unlike PGs. It seems therefore that adequate preparation of the myometrium is important for the action of oxytocin but not PGs. This is borne out by the findings that amniotic PG levels in patients carrying an anencephalic foetus contain low or undetectable PG levels before and during labour (Turnbull & Anderson,1978). PGs potentiate the action of oxytocin on the human pregnant uterus (Gillespie & Beazely,1972) in a manner similar to the rat.

The role of PGI_2 synthesis in the pregnant rat myometrium has been of great interest. The possibility that oxytocic drugs may stimulate uterine PGI_2 synthesis has been investigated. Oxytocin certainly stimulates rat pregnant myometrial PGI_2 synthesis as does angiotensin II, bradykinin and ergometrine (Williams, ElTahir & Seed, 1983). PLA_2 and AA also stimulate PGI_2 synthesis and this stimulation is inhibited by mepacrine suggesting that PLA_2 plays a part in this stimulation of PGI_2 synthesis. The effect of drugs on human myometrial PGI_2 synthesis has not been studied.

Histamine and relaxin inhibit rat pregnant myometrial PGI_2 synthesis (Williams et.al.,1983). Relaxin is a pituitary polypeptide released during late pregnancy (O'Byrne & Steinez, 1976) and is a pregnant uterine relaxant as is histamine (Kameswaran, Pennefather & West, 1962). β -adrenoceptor stimulation results in the relaxation of the rat uterus, being antagonised by propranolol and to a much lesser extent, practolol (Wasserman & Levy,1972). Salbutamol , a selective β_2-adrenoceptor stimulant, is used therapeutically to prevent or

reverse premature or oxytocin-induced labour in women (Lipschitz, Baillie & Davey, 1976; Liggins & Vaughan, 1973) since β_2 receptors are the predominant type (Bowman & Rand, 1980). Since the actions of salbutamol and 5-HT are receptor mediated, and receptor antagonists are readily available, these two drugs would be ideal to test the hypothesis that oxytocic and relaxant drugs may affect prostacyclin release by receptor mediated control of prostacyclin synthesis.

1.7) Prostaglandins and uterine blood flow

Uterine blood flow is greatly increased (by over 30 times) in pregnancy (Metcalfe, Romney, Swartwout, Pitcarim, Lethin & Barron,1959). There is a lack of reactivity to various dilators such as exogenous oestradiol, adenosine or bradykinin which has been likened to a "physiologic hyperaemia" (Clewell,1979). The uterine vascular bed constricts in response to adrenaline and noradrenaline with the same sensitivity in the non-pregnant and pregnant ewe (Barton, Killam & Meschia,1974) but dilates in response to angiotensin II (Terragno, Terragno, Pacholczyk & McGiff,1973) in the pregnant dog. Inhibition of PG synthesis decreases blood flow in pregnant dogs (Terragno, Terragno,Pacholczyk & McGiff, 1974), rabbits (Venuto, O'Dorisio, Stein & Ferris,1975) and ewes (McLaughlin, Brennen & Chez,1978). AA increases blood flow in pregnant dogs and this is inhibited by indomethacin (Terragno et. al.,1974)

Attempts to clarify the type of PG involved have been concentrated on the vasodilator, PGE_2 (Clark & Brody,1977) which does not totally restore blood flow after PG synthesis inhibition (Terragno et. al.,1974). Terragno has elucidated in retrospect that 6-oxo-$PGF_{1\alpha}$ is released into uterine venous blood in late pregnancy after separating 6-oxo-$PGF_{1\alpha}$ and PGE_2 which behaved similarly in the TLC system used

(Terragno & Terragno,1980). Thus flow may be completely restored on addition of PGI_2.

There is no data available on uterine blood flow comparing pregnant and non-pregnant women, however compromised systemic vascular reactivity in pregnant women has been described (Assali,1961). Angiotensin II has a reduced pressor activity which can be reversed by treatment with indomethacin (Everett, Worley, MacDonald, Chand & Gant, 1978), however the type of PG involved has not been elucidated.

Compromised uterine blood flow in preeclampsia is a major cause of foetal hypoxia and death. Systemic vasodilatation using hydralazine is an established treatment for preeclampsia (Berkow, 1977). The anti-hypertensive action of hydralazine in renal hypertensive rats has been shown to be dependant on PG synthesis since it is prevented by indomethacin (Cangiano, Rodriguez-Sargent & Martinez-Martinado, 1978). The action of vasodilators on myometrial PGI_2 would therefore be of interest.

1.8) Assay of PGI_2

Measurement of PGI_2 synthesis in uterine tissues is the aim of this study and thus a discussion of the assay methods available is necessary.

1.8 i) Biological assay

PGI_2 present in aqueous samples can be assayed by inhibition of platelet aggregation (Moncada et.al.,1976); relaxation of superfused bovine coronary artery (Moncada et.al.,1976) or the inhibition of platelet adherence to *ex vivo* blood-superfused tendon (Gryglewski,

Korbut, Ocetkiewicz, 1978).

1.8 ii) Radioimmunoassay

This technique has been reviewed by Granstrom (1981). As PGI_2 is unstable, anti-sera to its hydrolysis product 6-oxo-$PGF_{1\alpha}$ have been used. With radioimmunoassay (RIA) the inhibition of binding of radiolabelled 6-oxo-PGF_1 by test samples is measureable. This binding can be affected by compounds of similar structure as well as pH, proteins, lipid and alteration of ionic strength of the medium (Granstrom, 1981). Other detrimental factors may include interference from the separation methods, for example Amberlite XAD-2 degradation products compete for anti-PGF or anti-TxB sera. The specificity of anti-sera raised against 6-oxo-$PGF_{1\alpha}$ is in doubt since it cross reacts totally with 6-oxo-PGE_1 (Lee, McGiff, Housholder, Sun & Wong, 1979).

1.8 iii) Radiochemical assay

Labelled AA (^3H or ^{14}C) is incubated with the tissue and the products extracted, concentrated and separated by TLC. Specific solvent systems have to be used as PGE_2 and 6-oxo-$PGF_{1\alpha}$ have the same rf. values in many systems (Cottee, Flower, Moncada, Salmon & Vane, 1977 ; Sun, Chapman & McGuire, 1977). This technique detects radiolabelled metabolites of AA. Very little conversion is seen in chopped tissue as the labelled substrate has to compete with endogenously released AA for conversion by cyclo-oxygenase. This can be overcome by using the microsomal fraction of the tissue which concentrates synthetase activity and the liberated AA is discarded in the microsomal supernatant. However uterine microsomes require the added cofactors for expression of maximal activity (Downing & Williams, 1981). This can alter the profile of AA

metabolites formed and give a misleading picture of products synthesised in the whole cell.

This technique is time-consuming and relatively expensive to run.

1.8 iv) Gas chromatography-Mass spectrometry (GCMS)

This method requires derivitisation of 6-oxo-$PGF_{1\alpha}$ after extraction. The O-methyl-oxime, methyl ester of 6-oxo-$PGF_{1\alpha}$ is measured and the results obtained are extremely accurate and specific. The advantage of GCMS is the variety of compounds which can be assayed without confusion with interfering substances. The sensitivity is very high (50-250 pg) (Granstrom, 1981) and it is regarded as a reference method.

1.8 v) High Performance Liquid Chromatography (HPLC)

PGI_2 can be measured directly by this method as well as 6-oxo-$PGF_{1\alpha}$ (Hill, 1979; Wynalda, Lincoln & Fitzpatrick, 1979). However at present sensitivity is poor and furthermore the equipment is expensive.

1.8 vi) Assay choice

As already discussed (Section 1.3v) PGI_2 is metabolised to several compounds other than 6-oxo-$PGF_{1\alpha}$ and it is an assumption of radiochemical, radioimmunoassay and GCMS methods that PGI_2 is hydrolysed to 6-oxo-$PGF_{1\alpha}$. These methods, including HPLC, include lengthy extraction, purification and derivatisation procedures as well as substantial investment in capital equipment. Unless expensive kits are acceptable, radioimmunoassay also involves lengthy and costly preparative steps.

Bovine coronary artery bioassay requires equilibration for 4-7 hours to establish resting tone, and even though refrigerator storage can reduce this time (Dusting, Moncada & Vane, 1977) the recovery after PGI_2 induced relaxation is slow. There also may be interference from other PGs which contract the tissue (Omini et.al., 1977).

The platelet aggregation bioassay for the estimation of PGI_2 is the most convenient as it detects biological activity of the substance and does not measure the hydrolysis product. It is rapid, cheap and assays nanogram quantities of PGI_2. Interference by both PGD_2 and 6-oxo-$PGF_{1\alpha}$, which are antiaggregatory (Smith, Silver, Ingerman & Kossis, 1974; Lee, McGiff, Householder & Sun, 1979) can be abolished by careful control procedures.

1.9) Aims

1) To characterise the anti-aggregatory materials released by human placenta and myometrium in vitro.

2) To determine the effect of uterine stimulant and relaxant drugs on the release of antiaggregatory material from the pregnant rat myometrium.

3) To determine the effect of vasodilator drugs on the release of anti-aggregatory material from the pregnant rat myometrium.

4) To develop and characterise an assay for PLA_2 activity within the pregnant rat uterus.

2.0) METHODS - Studies on the production of anti-aggregatory activity by the pregnant human myometrium and placenta, and the production of PGI_2 by the pregnant rat myometrium

2.1) Preparation of human myometria and placentae

Lower segment human myometria were obtained at abdominal hysterotomy or elective caesarian section, before the onset of labour and before syntocinon was administered. Placentae were obtained from patients undergoing elective caesarian section and experiments were paired with myometrial samples taken from the same patient. Samples were placed in ice-cold Tyrode's solution and used within 2 hours. Samples were blotted dry and traces of blood and decidual tissue removed. The samples were then placed on ice in Tris-buffered saline (TBS : pH 8.0 ; 50 mM).

2.2) Preparation of pregnant rat myometrium and decidua

Female wistar rats (200-250 g, Bath University strain) were mated overnight and cervical plugs noted over the next two days. The presence of plugs were taken as evidence of successful fertilisation and this denoted as day 1 of pregnancy. Animals were killed on selected days of pregnancy, the uterine horns dissected out and placed into ice-cold Tris buffered saline (TBS : pH 8.0 ; 50 mM). The uterine contents were removed and the sites of nidation dissected out since they do not synthesise PGI_2 . Decidual tissue was separated from the myometrium by scraping with a microscope slide (Williams,1973). In some experiments decidual tissue was separated by teasing with a cotton bud. The weight of the decidua was calculated from the difference between that of the whole uterus and the separated myometrium.

In some experiments rats were anaesthetised with ether and the uterine horns exposed and kept moist with warm Krebs' solution. The arterial supply of one horn was clamped at the uterine and ovarian

arteries and the other horn perfused with warm (37°C) Krebs' solution via the femoral artery until no blood was visible (approximately 15 minutes). The vena-cava was sectioned to allow perfusate to escape.

2.3) Incubation conditions

Human myometrial and placental samples were suspended in TBS 50 % w/v. Rat myometrial samples from day 19 to 22 pregnant rats were blotted dry and suspended in TBS to give a 25% (w/v) mixture. The reaction was initiated by chopping the samples finely with dissecting scissors. Incubations were performed at 20°C for 15 minutes (rat myometrium and human placenta) and 30 minutes (human myometrium) . Samples were centrifuged at 2,000 g for 30 seconds in an Eppendorf microfuge (5412) to sediment all tissue from the incubation medium. The incubation media were aspirated and stored on ice. Portions of the incubation media were removed and their anti-aggregatory activities assessed.

2.4) Estimation of prostacyclin content of incubation media

i) Preparation of rabbit platelet-rich plasma

Blood was obtained from New Zealand White rabbits (2.5-4 kg) under ether anesthesia by cardiac puncture using a 16-gauge needle with silicone tubing attached. The blood was collected into plastic tubes containing 3.8 or 3.16 % (w/v) tri-sodiumcitrate (9 vol blood : 1 vol citrate). The tubes were gently inverted and centrifuged at 200g for 10 minutes at room temperature. The platelet-rich plasma (PRP) was then aspirated and pooled into one plastic tube. The PRP was then divided into 0.5 ml aliquots in 3 ml plastic tubes (Luckam PT/0944) or 0.2 ml aliquots in 0.5 ml tubes (Luckam LP/2). Tubes were maintained at room

temperature throughout the experiment.

ii) Inhibition of platelet aggregation

Each tube was placed in an aggregometer (Bryson, EEL or Malin dual channel), warmed at 37°C and stirred at 1100 rpm. The aggregometer output was connected to a 'JJ' pen recorder. The smallest dose of ADP that produced an irreversible aggregation was found (2.5-10 uM final concentration) and used for the duration of the experiment. Samples of incubation media (myometrial : 1.25-20 ul ; placental : 20-80 ul) were added to the cuvette one minute before ADP and the anti-aggregatory effect recorded. The PGI_2 content of the sample was estimated using a 2+2 doses assay against authentic PGI_2 and production expressed as ng/mg wet weight of tissue over the specified incubation time.

iii) Papaverine pre-treatment of PRP

In order to increase platelet sensitivity to PGI_2 some experiments were carried out using platelets pretreated with the phosphodiesterase inhibitor, papaverine. Samples (0.5 ml)of PRP were placed in an aggregometer as specified above. In test samples, papaverine, PGI_2 and ADP were added at consecutive one minute intervals, after one minute pre-incubation. Papaverine was omitted in the control group leaving a two minute pre-incubation period (see Table 1.)

Table 1: Time course of PGI_2 assay with or without papaverine.

Time Mins	Control	Test
0	pre-incubation	pre-incubation
1	pre-incubation	papaverine added
2	PGI_2 added	PGI_2 added
3	ADP added	ADP added

iv) Determination of assay coefficients of variation

Specimens of lower segment uterine tissue were obtained from three patients. These were pooled and a 50% w/v mixture incubated as above. The incubation mixture was then centrifuged after the 30 minute incubation period. The pH of the aspirated incubation medium was adjusted to pH 6.5 by the addition of 0.1N HCl, and placed in a water bath at 37°C for 60 minutes to destroy all endogenous PGI_2. The aspirate was then placed on ice, readjusted to pH 8.0 using 0.1 N NaOH, divided into four equal portions and stored at -20°C until required. PGI_2 was added to the aspirate to make a concentration of 250 ng/ml (prepared fresh each day) and kept on ice. Standard solutions were also made up fresh each day in TBS.

The PGI_2 content of each portion was then assayed against authentic PGI_2 using PRP from four different rabbits. In each experiment four 2+2 dose assays were performed for both control and pretreated groups. The coefficients of variation of the different concentrations assayed (x) were calculated from equation 1 :

Equation 1)

(standard deviation of x / mean of x) * 100 %

2.5) The effect of pH on the stability of human myometrial and placental anti-aggregatory activity

Myometrial and placental samples were incubated as normal. The incubation media were aspirated and the anti-aggregatory effect of a aliquots were assessed against ADP-induced aggregation of rabbit PRP. Other portions were then adjusted to pH 12.0 with 0.1N NaOH and maintained for 60 minutes on ice and reacidified to pH 8.0 with 0.1N HCl. The anti-aggregatory activity was then reassessed. Samples of authentic PGI_2, PGD_2 and PGE_2 were treated similarly.

Further experiments were undertaken to compare the stability of the human myometrial and placental activities at lower pH. Tissues were incubated as normal and the incubation media aspirated.The anti-aggregatory effect of a portion was assessed and other aliquots were adjusted to pH7.5 or pH 8.5 and then incubated at room temperature for 30 minutes. These portions were then reassessed for anti-aggregatory activity after readjustment to pH 8.0.

2.6) Effect of arachidonic acid and phospholipaseA_2 on human myometrial PGI_2 synthesis

These substances were added after pre-incubation of the tissues at 37^{o}C for ten minutes. The tissues were then chopped and incubated at room temperature for 30 minutes and the PGI_2 content of the media assayed.

2.7) Effect of uterine stimulants and relaxants on rat and human myometrial anti-aggregatory activity

To study the effects of drugs that affect uterine contractility the

methods of ElTahir and Williams (1980) were followed.

The tissue was pre-incubated with the drug for 10 minutes at 37°C prior to chopping. The tissue was then chopped after cooling and incubated at room temperature for 15 minutes (rat), 30 minutes (human myometrium and 15 minutes human placenta). The PGI_2 content was then determined . In control samples pre-incubation was carried out without drug addition.

In experiments using rat myometrium where antagonists to the stimulant or relaxant drugs were used, control samples were preincubated for 20 minutes with the antagonist prior to chopping whereas in test samples the agonist was added after 10 minutes pre-incubation with the antagonist and pre-incubation continued for a further 10 minutes. Samples were then cooled, chopped and incubated at 20°C for 15 minutes.

2.8) Elimination of carry-over effects of drugs used to influence PGI_2 synthesis

On incubation of myometrial tissue with drugs, the possibility that such drugs may affect platelet aggregability must be considered. To control for these effects, each drug or mixture was added with the PGI_2 standard in a volume and concentration equal to that in the unknown incubation medium being tested.

2.9) Assessment of adenosine diphosphatase (ADPase) activity

A series of experiments were carried out to determine if anti-aggregatory material(s) in the human myometrial and placental incubation media was due to ADPase activity. For this purpose, samples of the myometrial and placental incubates were heated to 37°C for 30 minutes to destroy endogenous PGI_2. ADP was then added to aliquots of this PGI_2-

free incubation medium (2.3 mM final concentration) and incubated at room temperature for 20 minutes. The aggregatory response induced by aliquots of these solutions were compared with that of ADP stock solution.

2.10) Effect of PGI_2 on isolated pregnant human myometrium

Segments were collected at caesarian section and placed in ice-cold Tyrode's solution. 2 X 0.5 X 0.1 cm sections were dissected from each specimen and mounted in a 10 ml organ bath and bathed in Krebs' solution at 37°C, oxygenated with 95% O_2 & 5% CO_2. Isotonic contractions were recorded under a 1g load using a 'JJ' pen recorder. Samples were equilibrated for 1 hour with washing every 15 minutes. Only those samples exhibiting spontaneity were used. Samples were challenged with 200ng/ml acetylcholine to ensure viability, prior to the addition of PGI_2. PGI_2 was added to the organ bath for a contact time of 8 minutes with 8 minutes recovery after washing.

3.0) METHODS - Assay of pregnant rat uterine PLA_2 activity

3.1) Liquid scintillation counting

A LKB 1215 liquid scintillation counter was used to detect radioactivity from tritiated (^{3}H) and carbon-14 (^{14}C). Quench curves were constructed using the external channels ratio vs efficency due to the ease with which it may be constructed with this equipment. All samples were counted for 10 minutes or to 100,000 counts, whichever occured first.

i) Scintillation cocktails

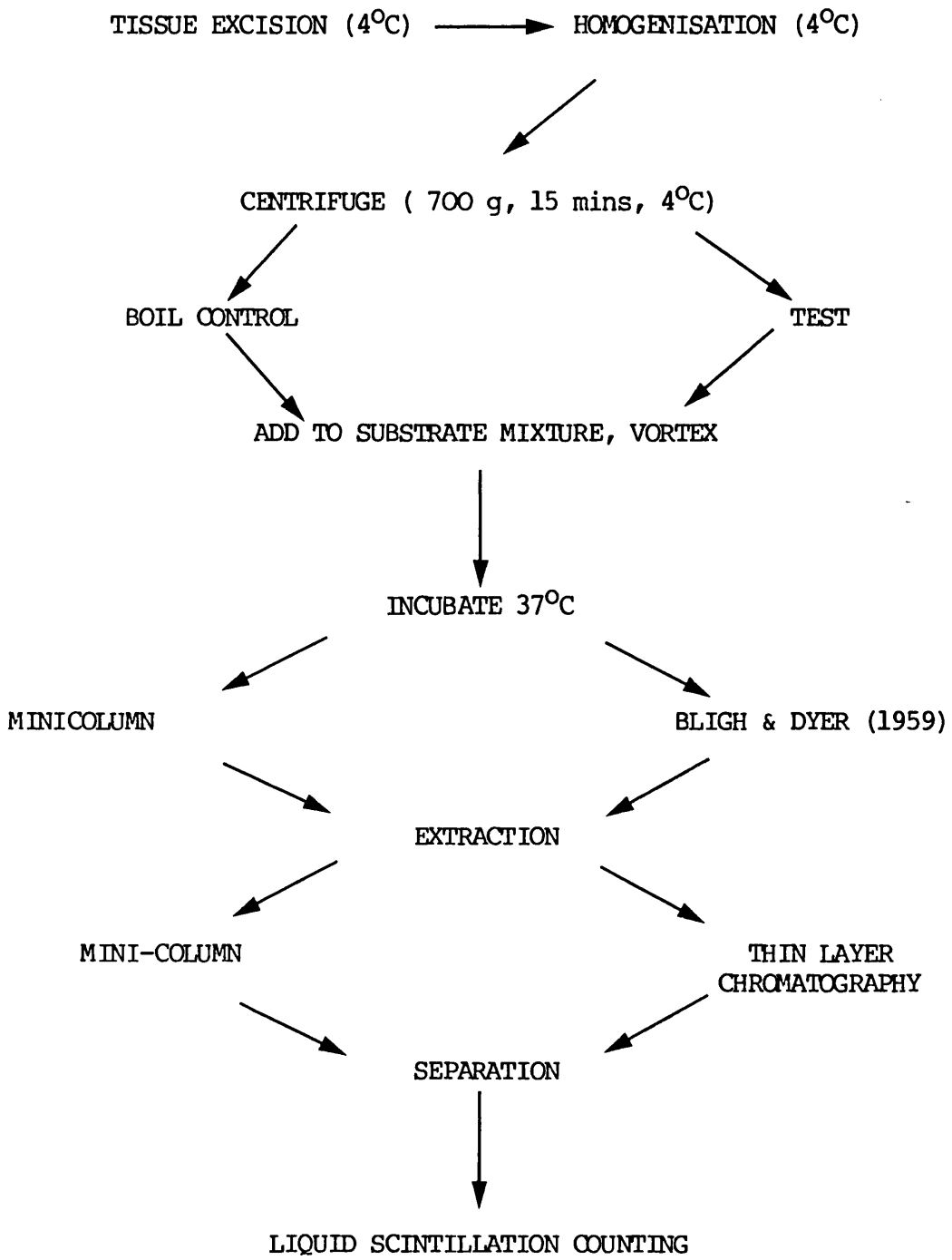
'Aqua Luma' (Lumac Systems Inc.) was used for determining activity in aqueous samples and a secondary scintillant (2,5-diphenyloxazole, 0.3g; 1,4-di(2-(5-phenyloxazolyl))benzene, 0.2g; Triton-X114, 25ml and xylene, 75ml) for all other samples.

ii) Construction of quench curves

Quench curves were constructed using capsules containing known amounts of ^{3}H (LKB cat N^{o} 1210-120 : 101100 dpm) and ^{14}C (LKB cat N^{o} 1210-122 : 121100 dpm). 8ml of carbon tetrachloride was added to 100ml of scintillant and diluted for each point. Quench curves were constructed for ^{3}H alone, ^{14}C alone and ^{3}H with ^{14}C for dual labelling. All samples contained 4ml of quenched cocktail in plastic vials and loaded into the counter which had been programmed to count and construct the quench curve. The data was stored in the counter memory and the efficiency calculated automatically from these curves for all subsequent experiments.

For dual labelled samples the spillover of counts from the high energy channel (^{14}C) to the low energy channel (^{3}H), and vice versa, is

Diagram 1) Flow diagram illustrating the sequence of preparation of incubation media, extraction and separation of oleic acid from dPC for the assay of rat uterine PLA_2.



calculated. The disadvantage of this procedure is that if there is very low, or negligible, activity in one channel and high activity in the other, then the amount of spillover calculated is subtracted and results in a negative value in that channel with low activity. Therefore this method was only used for the calibration of mini-columns to illustrate separation of these two labels.

3.2) Preparation of uterine tissue

Rat pregnant whole uterus was either placed in one of three ice-cold media:

a) Tris-HCl buffer (100mM ; pH7.5 ; 5mM $CaCl_2$)

b) Tris-HCl buffer (100mM ; pH7.5 ; 5mM $CaCl_2$) with tween-80 (0.1% v/v)

c) Sucrose solution (0.25 M)

Tissue was added to make 25% (w/v) mixtures and chopped finely before homogenisation on ice with an 'Ultra Turrax' homogeniser for 20 seconds. The homogeniser blade was kept in ice-cold buffer until immediately before use. The homogenate was then centrifuged at 700g in a MSE 'Chilspin' for 15 minutes at $4^{o}C$ to sediment nuclei and large elements of cell debris. The supernatant was then aspirated and stored on ice.

3.3) Synthesis of 1'-oleoyl,2'[^{3}]Holeoyl-phosphatidylcholine (^{3}H-dPC)

^{3}H-dPC was kindly donated by Dr. G.J.Blackwell (Wellcome research laboratories). Synthesis was carried out using the method of Blackwell, Duncombe, Flower, Parsons and Vane (1977).

2mg carrier oleic acid and ^{3}H-oleic acid (<10 Ci/mMol) with 20mg of 1-acyl-phosphatidylcholine were added to a reaction flask and organic solvents removed. The mixture was sonicated with 150ml phosphate buffer (0.1M ; pH 7.5) and 200ul adenosine triphosphate (ATP), 200umols $MgCl_2$, 2uMols Co-enzyme-A, and 3ml 0.25M sucrose containing rat liver microsomes (11g rat liver wet weight homogenised in 9ml sucrose). Incubation was carried out at 37^{o}C with shaking and further equal amounts of ATP, $MgCl_2$, CoA and microsomes added at 30 minutes and 120 minutes. After 3 hours incubation, the mixture was extracted with chloroform : methanol (2 : 1) and the chloroform phase dried over anhydrous $MgSO_4$. The concentrated extract was applied to a column of 16g silicic acid with 4g Hyflo, slurried with 10 % (v/v) methanol : chloroform. Unreacted fatty acids were eluted with 10% (v/v) methanol : chloroform and ^{3}H-dPC with methanol : chloroform (40 : 60). 50ml fractions were collected and the radioactive peak isolated.

3.4) Substrate preparation and incubation

Amounts of ^{3}H-dPC (sp.act. <10 Ci/mMol) and ^{14}C-oleate (sp.act. 59.9 mCi/mMol)) were dispensed as described in the relevant section.

^{3}H-dPC was placed in plastic Sarstedt tubes (72.690) and dried under nitrogen gas with unlabelled 1,2-dioleoyl-phosphatidylcholine (dPC; concentrations as specified). Crude enzyme (in Tris-HCl [solution 1] ; or Tris-HCl with Tween-80 (0.1%) [solution 2] was added and

vortexed for 1 minute. The solutions were incubated at 37°C for the times specified.

The third medium (solution 3) was adapted from the method of Dey, Hoversland and Johnson (1982). ^{3}H-dPC and dPC was dried in glass tubes under nitrogen. Solution 3 was prepared with N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES : 50mM, pH and calcium concentration as specified), HEPES with 0.2% Triton X-100 and the uterine homogenate in sucrose in the ratio 5 : 3 : 1.7 . Thus if the total incubation mixture had a volume of 300ul, this would contain 150ul HEPES, 100ul HEPES with Triton X-100 (0.2%) and 50ul of uterine homogenate. Before the homogenate or blank sucrose was added, the mixture was placed in boiling water for 1 minute, immediately cooled in iced water, and bovine serum albumin (BSA: 1mg/ml final concentration) was added to bind released ^{3}H-oleic acid and thus prevent reacylation. The mixture was then sonicated at 4°C with 20 second bursts separated by 5 seconds for two minutes in order to create Triton micelles. Solution 3 was then incubated at 37°C for varying periods.

3.5) Solubility of 1,2-dioleoyl-phosphatidylcholine (dPC) and oleic acid in the three substrate solutions.

The three incubation mixtures were prepared as above, each 150ul sample containing 50nMols (i.e. 333uM) dPC and ^{3}H-dPC (10,000-25,000 DPM) at pH 7.5 without uterine tissue but with 0.25M sucrose substituted for the uterine homogenate in solution 3. A further group of mixtures were prepared without dPC and ^{3}H-dPC (10,000 - 25,000 DPM) but with ^{14}C-oleate (10,000 - 20,000 DPM). After preparation, aqueous samples were pipetted into scintillation vials and taken up in 4ml Aqua Luma, the activity measured and compared with controls dispensed directly into the

scintillation vials and evaporated under nitrogen. The percentage loss was then calculated .

3.6) Column chromatography

3.6 i) Preparation of minicolumns

Mini-columns were prepared according to the method of Cosentino and Le Grande (1981). Glass disposable Pasteur pipettes (130mm X 5mm) were plugged with glass wool and packed to a height of 5cm with silica gel (Sigma 100-200 mesh) suspended in hexane : dioxane : glacial acetic acid (Solvent 1: 70 : 30 : 1, v/v/v). The silica gel was either heated to 100^{o}C for 1 hour or not heated, and was equilibrated in solvent 1 for 0-14 days before use. A negative pressure fume cupboard was used throughout column preparation and use.

3.6 ii) Extraction and separation of ^{3}H-dPC and ^{14}C-oleic acid from aqueous samples using mini-columns

The advantage of this procedure is that both extraction and separation are achieved within the same step.

150ul aliquots of solution 2 containing ^{3}H-dPC (333uM : 75,000 DPM pH 7.5) and ^{14}C-oleic acid (40,000 DPM) and solution 3 containing dPC (333uM : 48,000) and ^{14}C-oleic acid (40,000 DPM) were applied directly to the columns containing 100-200 mesh silica gel which had been equilibrated for 7 days in solvent 1. 1 ml of solvent 1 was then immediately added. This was gently forced into the column packing by applying pressure via a pipette bulb placed over the column. The 1ml eluate was collected into a scintillation vial. Five further 1ml aliquots of solvent 1 were added to the column and successive eluates

(containing ^{14}C- or ^{3}H-oleic acid) collected (for each 1ml sample). The ^{3}H-dPC was then eluted by the addition of six 1ml aliquots of solvent 2 (Chloroform : methanol : water; 65 : 35 : 4 , v/v/v) and collected as before. Both solvents 1 and 2 were dried down under nitrogen gas in a water bath at 37°C and the scintillation cocktail added.

Column retention of ^{3}H-dPC and ^{14}C-oleic acid was calculated by comparing the total radioactivity eluted with 150ul of the same sample taken up in Aqua Luma. Dual labelled scintillation counting was used for these procedures.

3.7) Thin layer chromatography

3.7 i) Extraction of dPC and oleic acid from aqueous samples

a) Organic solvents and pH

150ul samples of substrate solution 3 (pH7.5 ; 5mM $CaCl_2$;333uM dPC) containing either 3,000 DPM of ^{3}H-dPC or 3,000 DPM of ^{14}C-oleic acid were adjusted to pH 3.0 or 11.0 by the addition of 0.1N HCl and 0.1N NaOH. These samples were vortexed with 1ml each of the following solvents: chloroform, dichloromethane, n-heptane, diethylether and butan-1-ol. All were compared to 150ul aqueous samples taken up in Aqua Luma.

b) Method of Bligh and Dyer (1959)

150ul samples of solution 3 (pH 7.5 ; 5mM $CaCl_2$; 333uM dPC) containing 4,5000 dpm ^{3}H-dPC and 3,500 DPM ^{14}C-oleic acid were prepared and added to tubes containing 188ul chloroform and 375ul methanol and vortexed, forming a miscible chloroform : methanol : water system (ratio 1:2:0.8 respectively). A further 188ul of chloroform was added and

vortexed followed by 188ul of distilled water which separated the solution into chloroform and methanolic layers. The lower (chloroform) layer was aspirated into scintillation vials and the methanolic layer further washed with one volume of chloroform. It was found that the addition of 50ul of methanol to the chloroform layer ensured phospholipid solubility. The chloroform was dried down and scintillation cocktail added. The results were compared to 150ul of corresponding aqueous samples added directly to Aqua Luma and % recovery calculated. If the sample was to be applied to TLC plates, the sample was dried in Sarstedt tubes to approximately 30ul and applied to the TLC plates.

3.7 ii) Preparation of TLC plates

Whatman TLC plates (LK6D) were heat activated at 100°C for 1 hour prior to use, and stored in a dessicator cabinet until samples were applied to the plates. Plates were run in chloroform : methanol : glacial acetic acid : water (50 : 15 : 4 : 2 , [Solvent 3]). The tanks were equilibrated with the solvent system for 1 hour before use.

3.7 iii) Separation of dPC and oleic acid

^{3}H-dPC (400,000 DPM in chlorofrom : methanol [60:40]) and ^{14}C-oleic acid (200,000 DPM in toluene) were spotted onto separate lanes and run to 10cm. The plates were then scanned using a Berthold TLC scanner (2X2 mm window) to localise peaks of activity. 1mg of 1~lysophosphatidylcholine was spotted onto one lane and the plate run as above and visualised under iodine vapour.

3.7 iv) Elution of dPC and oleic acid from TLC zones

^{3}H-dPC (50 nMols [140,000 DPM], equivalent to a 150ul aqueous sample containing 333uMolar dPC) and ^{14}C-oleic acid (80,000 DPM) were applied to separate lanes of the TLC plates and developed to 10cm. The zones containing ^{3}H-dPC (origin - 8cm) and ^{14}C-oleic acid (8 - 12cm) were scraped into scintillation vials. Scintillation cocktail was added with or without 1ml methanol and the % recovery compared to samples added directly to the scintillation vials. The 30ul chloroform extracts of incubation media were applied to separate lanes of the TLC plates and developed to 10cm. The zones containing the ^{3}H-dPC and released ^{3}H-oleic acid were also scraped into scintillation vials.

3.8) Protein assay

Protein content of rat uterine incubation media were assayed using an adaptation of the method of Lowry, Rosenbrough, Farr and Randall (1951). Since HEPES interferes with the assay , rat uterine homogenates were added to saline in the same ratio (17 % homogenate v/v) as the incubation mixtures. The assay was carried out using the following solutions:-

A : 5 % w/v $CuSO_4$

B : 10 % w/v Na/K-Tartrate

C : 2 % w/v Na_2CO_3 in 0.1N NaOH

D : 1 vol Folin-Ciocalteau reagent
& 2 vol distilled water

E : 1ml A & 1ml B & 8ml water

F : 1ml E & 50ml C

Solutions E and F were prepared immediately before use and

solutions A to D stored at $4^{o}C$.

Incubation media were prepared containing 0.2ml samples (diluted 1 : 4 or 1 : 8 as appropriate), 2.0 ml F and 0.2 ml D. The samples were mixed well and allowed to stand for 30 minutes at room temperature in the dark. The absorbance was read at 650nM against a reagent blank and protein concentrations calculated from a calibration curve prepared using purified bovine serum albumin as standard.

3.9) Calculation of phospholipase-A_2 activity

The amount of ^{3}H-oleate released from ^{3}H-dPC was calculated by subtracting the ratio of DPM in the oleic acid fraction : total DPM of the sample boiled blank sample (B), from the ratio of oleic acid : total DPM of the test sample (T).This ratio of oleic acid : dPC was then used to calculate the absolute amount of oleic acid (nMols) released by multiplication by the total amount of dPC present (nMols). nMols of oleic acid released was expressed as a function of the total protein content in mg (P) in the tube per hour of incubation. (Equation 2).

Equation 2)

$$((T - B) \times \text{nMols dPC}) / P \times \text{time} = \text{nMols oleic acid released } mg^{-1} \text{ hour}^{-1}$$

3.10) Action of authentic phospholipase-A_2 on 1-oleoyl,2-([^{3}H]-oleoyl)-phosphatidylcholine

Authentic porcine pacreatic PLA_2 (5 U/ml) was incubated with 150ul samples of solution 3 containing 333uM dPC (50,000 DPM, minicolumns : 400,000 DPM, TLC) at $37^{o}C$ for 30 minutes. Samples were extracted and separated using minicolumns or the method of Bligh & Dyer followed by

TLC. Samples were then compared by liquid scintillation counting and the TLC samples scanned using a Berthold TLC scanner (2X2 mm window).

3.11) Time course of ^{3}H-oleic acid release and the comparison of separation of dPC and oleic acid by mini-column and TLC after incubation with rat uterine homogenate

Solution 3 (pH 7.5 ; 5mM $CaCl_2$) was prepared by vortexing 1ml HEPES and 0.66ml HEPES-Triton X-100 (0.2%) with ^{3}H-dPC (15,000 DPM per 150 ul of final incubation mixture) and dPC (to make 333uM). The solution was vortexed with 0.33ml of rat uterine homogenate (19 day pregnant) and incubated at 37^{o}C . 150ul samples were taken in duplicate from 0-60 minutes and extracted (Bligh & Dyer 1959 ; see section 3.7 i. b) for application to TLC plates for separation .

When TLC and mini-column separation were compared, paired 150ul samples were placed on mini-columns and extracted for TLC. Samples containing boiled homogenate were used as controls.

3.12) Incubation of rat uterine homogenate with phospholipaseA_2 inhibitors

Day 18 and 19 pregnant rat uterine homogenates were incubated in solution 3 at pH 7.5 containing 5mM $CaCl_2$ and 333 uM dPC (5,000 DPM per 150 ul of final incubation mixture). Mepacrine or tetracaine was added to solution 3 prior to the addition of the homogenates to make 6.77 uMolar and 1 mMolar final concentrations. Duplicate 150ul samples were taken over 60 minutes and compared with boiled protein controls.

3.13) Protein dependance

Three substrate mixtures (solution 3, pH 7.5 ; 5mM $CaCl_2$) were prepared by vortexing 3.0ml HEPES and 2.0 ml HEPES-Triton X-100 with ^3H-dPC (25,000 DPM per 150 ul of final incubation mixture) and dPC (333uM) and sonicated. To three 1.5ml aliquots of substrate solution was added either a) 0.75 ml boiled day 18 pregnant uterine homogenate as blank, or b) 0.75 ml uterine homogenate, or c) 0.38 ml uterine homogenate with 0.38 ml sucrose solution. This was then vortexed and incubated at 37°C . Duplicate samples were taken at 5, 10 and 20 minutes and extracted (Bligh & Dyer,1959) for separation by TLC.

3.14) pH dependence

Aliquots of solution 3 (5mM $CaCl_2$; 333 uM dPC [25,000 DPM per 150ul of final incubation mixture]) were prepared over a range of 6.75-8.5 by the addition of volumes of 0.1N HCl or 0.1N NaOH prior to vortexing with uterine homogenate. Duplicate 150ul samples were taken at 5, 10 and 20 minutes and the mean pH optimum found for two day 20 pregnant rat uteri.

3.15) Calcium dependence

Solution 3 (pH 8.0 ; 333uM dPC [25,000 DPM per 150ul of final incubation mixture]) was prepared by vortexing 300ul HEPES and 200ul HEPES-Triton X-100 with calcium in concentrations from 0-10 mMolar. 100ul of rat uterine homogenate (20 day pregnant) was added to each tube and incubated over 20 minutes. 150ul samples were taken in duplicate at 5, 10 and 20 minutes and extracted as before for separation by TLC. Samples containing boiled protein were used as controls.

3.16) Substrate dependence

Solution 3 (5mM $CaCl_2$; pH 8.0; ^{3}H-dPC 25,000 DPM per 150ul sample of final incubation mixture) was prepared by vortexing 300ul HEPES and 200ul HEPES-Triton X-100 with 50, 75, 100, 200, 400 and 800 nMols dPC with ^{3}H-dPC in glass tubes (to make 41.6 - 1332 uM final concentrations) and sonicated. 100ul of rat uterine homogenate (20 day pregnant) was added to each tube and incubated over 20 minutes. 150ul samples were taken at 5, 10 and 20 minutes and extracted as before for separation by TLC. Samples containing boiled protein were used as controls.

3.17) Statistics

A statistics program developed by Tallarida and Murray (1981) was modified, loaded onto disc and run using a "Superbrain II" microcomputor (Intertec Data Systems). All statistical procedures were carried out using this program. The paired and unpaired 't' tests were used to compare test groups with a single control, and Dunnet's test to compare several test groups with a single control. For figures 18 and 19 (sections 4.13 & 4.14) results were compaired by paired 't' test after $\log_{10}$ transformation. The Lineweaver-Burke plot was used to calculate Vmax and Km of the rat uterine PLA_2 (section 6.15).

3.18) Drug and solutions

a) Sources of Chemicals and drugs

Arachidonic acid	Sigma
Adenosine di-phosphate	Sigma
Angiotensin amide	Ciba

Dioleoyl-phosphatidylcholine	Sigma
2'5'-diphenyloxazole	Sigma
1,4-di(2-(5-phenyloxazoyl))benzene	Sigma
Dipyridamole (Persantin)	Boehringer Ingleheim
Ergometrine maleate	Sandoz
Forskolin	Calbiochem
Hydralazine HCl	Sigma
N-2-hydroxyethyl piperazine-N'-2-ethane-sulphonic acid	Sigma
Indomethacin	Merck, Sharpe & Dohme
Mepacrine HCl	Sigma
^{3}H-oleic acid	Amersham International
^{14}C-oleic acid	Amersham International
1~-oleoyl phosphatidyl choline	Sigma
Oxytocin (synthetic)	Sandoz
Papaverine HCl	Sigma
Phospholipase A_2 (porcine pancreas)	Sigma
Prostaglandin D_2	Sigma
Prostaglandin E_2	Sigma
Prostacyclin (PGI_2)	Wellcome Labs
Salbutamol (Ventolin)	Allen & Hanbury's
Sodium carbonate	B.D.H.
Sucrose	B.D.H.
Silica Gel (Mesh 100-200)	Sigma
Tetracaine HCl	Sigma
Tris (hydroxymethyl) aminomethane	Sigma
Triton X-100	Koch-Light Labs

Triton X-114	Sigma
Tween-80	Sigma

b) Sources of Solvents

Acetic acid (Glacial)	Fisons (AR)
Chloroform	B.D.H.
Diethyl ether	Fisons (AR)
1,4 Dioxan	Fisons (AR)
n-Hexane	Fisons (AR)
Methanol	Fisons (AR)
Toluene	B.D.H.
Xylene	B.D.H.

c) Solutions

Krebs' solution

NaCl, 6.9 ; KCl, 0.35 ; $CaCl_2$, 0.55 ; $KHPO_4$, 0.16 ; $MgSO_4.7\ H_2O$, 0.16 ; Glucose, 1.0 and $NaHCO_3$, 2.1 (g/dm^3).

Tyrode's solution

NaCl, 8.0 ; KCl, 0.2 ; $MgCl_2$, 0.1 ; $NaHPO_4$, 0.05 ; $NaHO_3$, 1.0 ; $CaCl_2$, 0.2 and glucose, 1.0 (g/dm^3).

Arachidonic acid and indomethacin

AA was stored in benzene until required. Samples were then blown dry under dry nitrogen gas and resuspended in TBS and dissolved with an equivalent amount of $NaHCO_3$. Indomethacin was added dry to TBS and dissolved in a similar manner.

Dipyridamole

Dipyridamole was dissolved initially in 66% absolute ethanol and diluted to the required concentration.

Hydralazine

Hydralazine was dissolved in TBS, kept on ice and used within one hour.

Other drugs

All other drugs were made to the required concentrations in TBS unless otherwise specified and stored on ice throughout the experiments.

Prostacyclin

Prostacyclin-Na was supplied by Wellcome Laboratories as the solid and stored at -20°C in 0.1 N NaOH (pH 11.0 , 10mg/ml). Aliquots of this solution were diluted to 100ug/ml in 100mM Tris buffer (pH 11.0) and stored at -20°C. From this, standard solutions were prepared fresh each day in 50 mM Tris buffer (pH 8.0) and were stored on ice throughout the experiment.

PGI_2 was also prepared in ungassed Krebs' solution and stored on ice for application to human pregnant myometrium *in vitro* (as described in section 2.10).

4.0) RESULTS - Studies on the production of anti-aggregatory material by the pregnant human myometrium and placenta

4.1) The effect of papaverine on ADP-induced platelet aggregation

In order to increase the sensitivity of platelets to the anti-aggregatory influences of PGI_2, PRP was pretreated with the phosphodiesterase inhibitor papaverine. Initially the effect of papaverine on ADP induced platelet aggregation was observed in order to to establish a threshold concentration of papaverine which could be used for this purpose.

Papaverine was added to PRP after 1 minute's stirring at 37°C and incubated with the platelets for 2 minutes before ADP addition. Papaverine exhibited dose-related anti-aggregatory activity (see figure 4). At 50uM the inhibition was 6.26 +/- 1.4% (mean +/- s.e.m.; n=6), 100uM (14.1 +/- 1.9%); 200uM (32.9 +/-11.1%), 400uM (72.1 +/- 10.4%) and 800uM (97.4 +/- 1.9%) inhibition of platelet aggregation). 50uM and 100uM papaverine thus exhibited little anti-aggregatory activity. These doses were therefore used to study their interaction with PGI_2.

4.2) The effect of papaverine on the inhibition of platelet aggregation by PGI_2

Platelets were incubated and stirred for one minute prior to the addition of papaverine. PGI_2 was added after 1 minute incubation with papaverine and aggregation induced with ADP 1 minute later. Dose response curves of the anti-aggregatory activity of PGI_2 were constructed alone or in the presence of 50, 100 and 200uM papaverine (see figure 5). The concentration of PGI_2 required to inhibit platelet aggregation by 50% (ID_{50}) was reduced from 7.2 +/- 0.7 ng PGI_2 /ml of PRP (mean +/- s.e.m.; n=4) to 3.9 +/-0.4 by 50uM papaverine; to 2.2 +/- 0.6 ng/ml by 100uM and to 1.5 +/- 0.4 ng PGI_2 /ml by 200uM papaverine. The dose of PGI_2 producing a 25% inhibtion of aggregation (i.e. ID_{25})

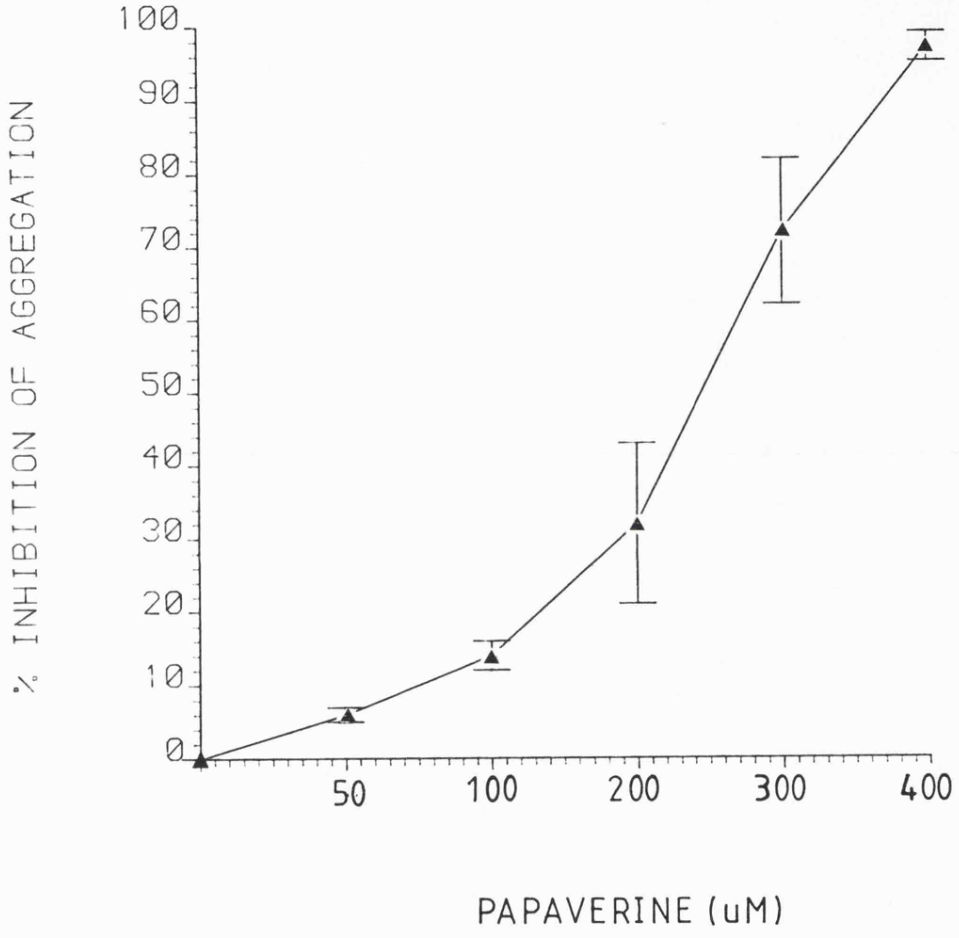


Figure 4) The effect of papaverine on ADP-induced platelet aggregation of citrated rabbit platelet-rich plasma. Papaverine (50 - 400uM) exhibited a dose-related inhibition of ADP-induced platelet aggregation. Triangles and vertical bars indicate mean +/- s.e.m.(n=6).

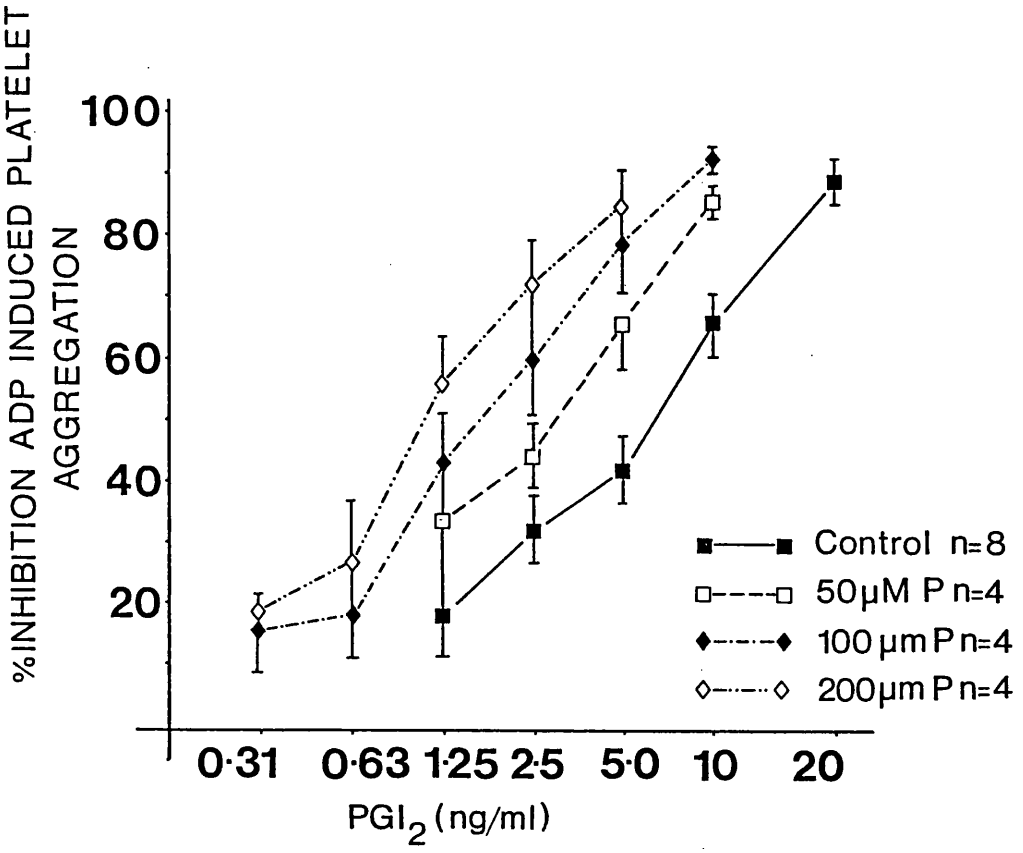


Figure 5) The effect of papaverine (50,100,200 uM) on the anti-aggregatory action of PGI_2 using ADP-induced aggregation of rabbit platelet-rich plasma. Papaverine potentiated the effect of PGI_2 in a dose related fashion. Points and vertical bars represent means +/- s.e.m.

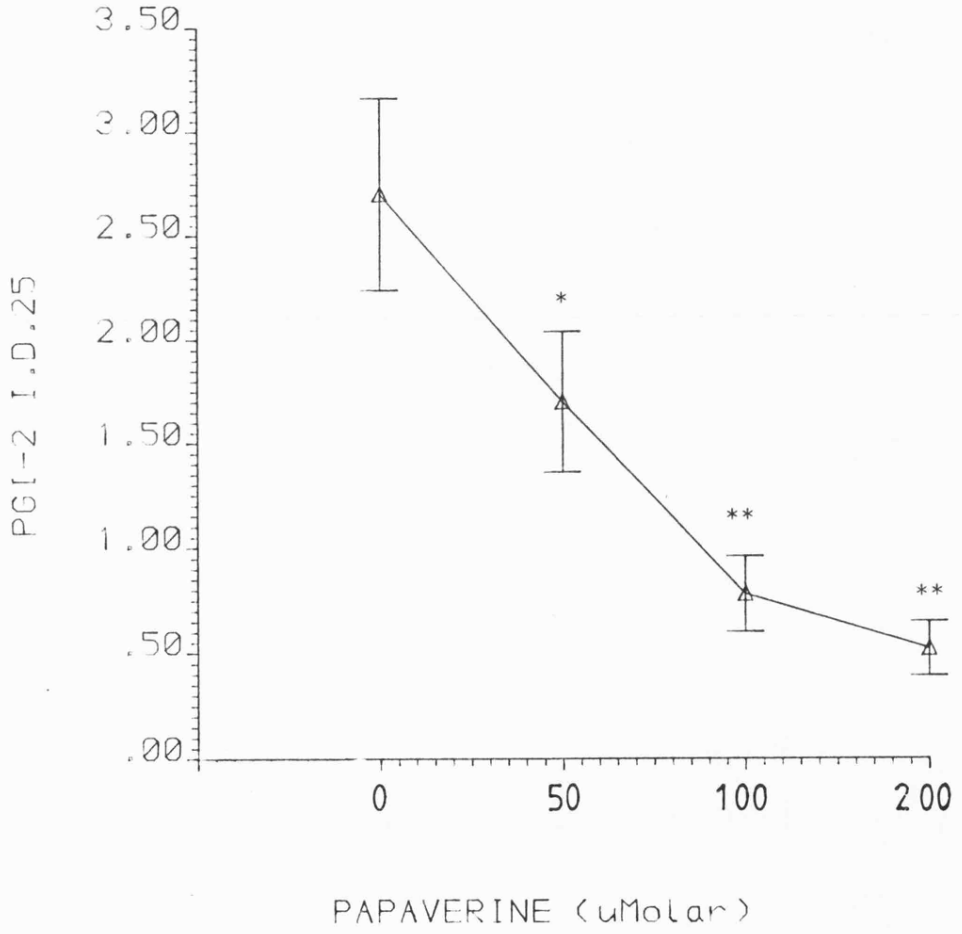


Figure 6) The effect of papaverine on the dose of PGI_2 required to inhibit ADP-induced platelet aggregation by 25% (ID_{25}). Papaverine reduced the ID_{25} in a dose-related fashion. Points represent mean +/- s.e.m. Significant differences are indicated (* $P<0.05$: ** $P<0.01$, n=6).

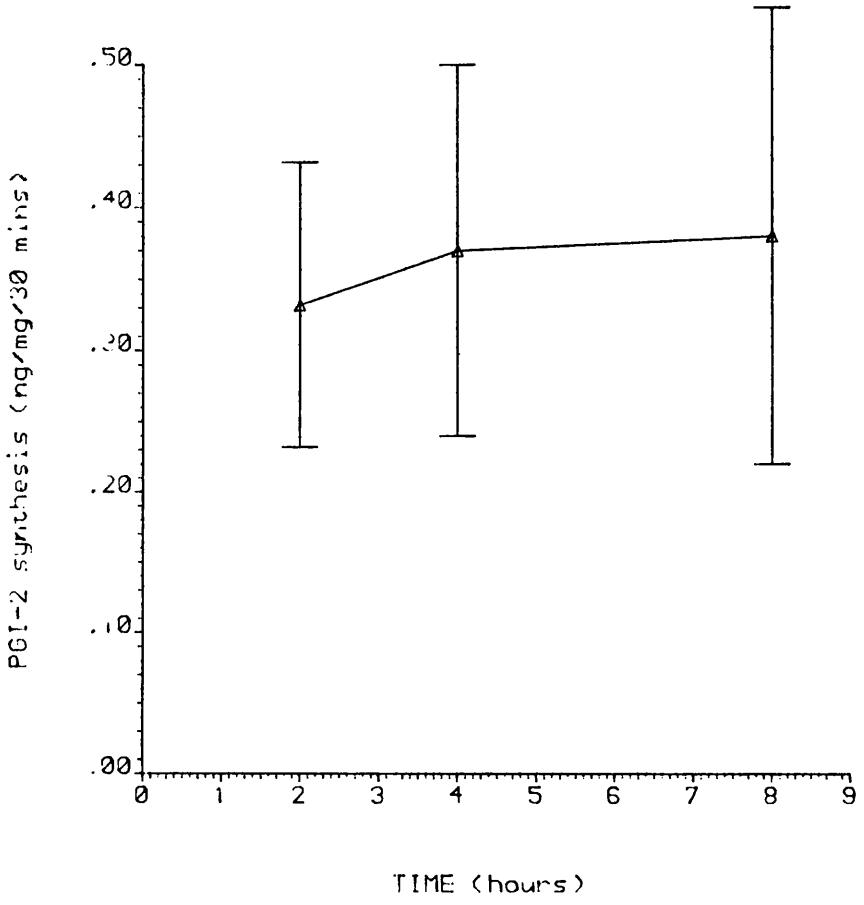


Figure 7) The effect of time interval between caesarian section and incubation on human pregnant myometrial release of anti-aggregatory activity. Although there was no difference in the mean release , the s.e.m. increased at 8 hours but not significantly. Points and vertical bars indicate mean +/- s.e.m.

was reduced from 2.69 +/- 0.5 ng PGI_2 /ml to 1.68 +/- 0.34 by 50uM papverine; 0.78 +/- 0.2 (100uM) and 0.51 +/- 0.1 ng PGI_2/ml by (200uM) (see figure 6). The slopes of the curves for PGI_2 alone or in the presence of the different doses of papaverine were not significantly different (test for parallelism; Tallarida & Murray, 1980)

4.3) The effect of time interval between delivery and start of incubation on the anti-aggregatory material released from human myometrium

Previous work in this laboratory has shown that maximal release of anti-aggregatory material is achieved after chopping and 30 minutes incubation at room temperature . All human myometrial samples were therefore treated in this manner to generate anti-aggregatory material.

Figure 7 shows that the release of anti-aggregatory material from three human myometria (weeks 38, 39, 40) is constant when stored up to 8 hours on ice in Tyrode's solution. However the standard error was noted to increase over time, though not significantly, and therefore all samples were used as soon as possible after section (usually within 2 hours of collection).

4.4) Preparation of PGI_2-free incubation medium from human pregnant myometrial tissue

PGI_2-free medium was prepared in order to test the effect of papaverine on authentic PGI_2 under the experimental conditions which would be used.

After chopping and subsequent incubation, human pregnant myometrium produced anti-aggregatory activity. The medium was incubated

for 10 minutes and 60 minutes at pH6.5 at 37°C This activity was markedly reduced after 10 minutes incubation and abolished after 60 minutes incubation (Figure 8). Pretreatment of platelets with papaverine (100uM) confirmed that no residual activity remained, and showed that PGI_2-free medium does not affect ADP-induced platelet aggregation in the presence of papaverine.

4.5) The effect of PGI_2-free incubation medium from human myometrium on the anti-aggregatory activity of PGI_2

The possibility that the PGI_2-free aspirate may alter platelet sensitivity to PGI_2 was investigated. PGI_2 (2.5ng) was added one minute prior to ADP (5uM) with or without 20ul of PGI_2-free human myometrial incubate. PGI_2 (1.25ng) was also added with or without 20ul of human PGI_2-free myometrial incubate to PRP pretreated with papaverine (100uM). Figure 9 shows that the anti-aggregatory activity of authentic PGI_2 remained the same in the presence of PGI_2-free human myometrial incubate whether the platelets were pre-treated with papaverine or not. Thus authentic PGI_2 may be used as a standard without the concomitant addition of PGI_2-free incubate.

4.6) Assay coefficients of variation

PGI_2 was added to PGI_2-free human myometrial incubate to make a concentration of 250ng/ml and assayed by a 2+2 doses assay. This was compared using PRP from four rabbits with and without papaverine pre-treatment and the assay repeated four times within each sample (see figure 10 for example). The assays without papaverine and with papaverine (100uM) gave total means of 242.4 +/- 0.4 and 262.3 +/- 0.4 respectively. Intra-assay coefficients of variation with and without

Control

	Assay-PGI2ng/ml					
Animal	1	2	3	4	$\bar{x}$	coeff
A	206·2	261·2	272·1	271·8	252·8	12·5%
B	258·4	243·2	237·0	231·5	242·5	4·8%
C	223·2	230·7	263·3	252·3	242·4	7·7%
D	272·7	216·9	241·8	194·8	231·6	14·5%
$\bar{x}$	240·1	238·0	253·6	237·6		
coeff	12·8%	7·9%	6·6%	13·9%		

Papaverine Pretreated

	Assay-PGI2ng/ml					
Animal	1	2	3	4	$\bar{x}$	coeff
A	199·8	277·5	295·0	310·0	270·6	18·1%
B	194·6	257·5	262·0	272·0	246·5	14·3%
C	227·9	295·0	315·6	241·0	269·9	15·6%
D	264·0	256·0	270·6	257·6	262·1	2·5%
$\bar{x}$	221·6	271·5	285·8	270·2		
coeff	14·4%	6·8%	8·5%	10·9%		

Table 2) Coefficients of variation for the assay of PGI_2 (250ng/ml placed in PGI_2 free human myometrial incubate) by the inhibition of ADP - induced platelet aggregation with or without pretreatment with 100uM papaverine. Samples were assayed using PRP from four rabbits (A-D) and assessed four times per rabbit (1-4).

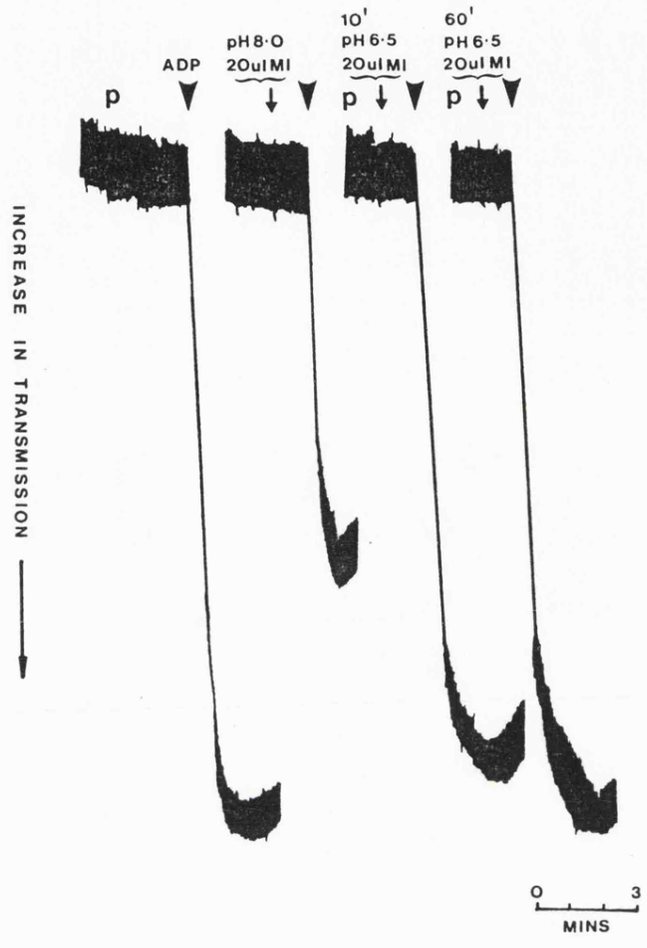


Figure 8) Preparation of PGI_2-free medium after incubation of human pregnant myometrial tissue. Myometrial incubate (MI) was incubated at 37°C for 10 and 60 minutes at pH 6.5 and tested for anti-aggregatory activity. Aggregation of citrated rabbit platelet-rich plasma was induced by ADP (5uM, ▼) . Papaverine (100uM, p) was used throughout . Incubation for 60 minutes at 37°C abolished all anti-aggregatory activity.

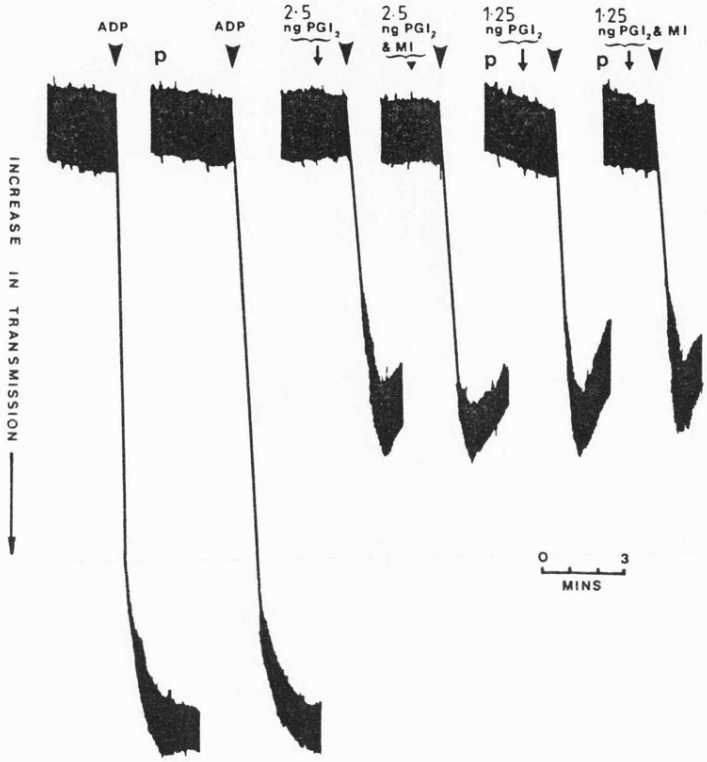


Figure 9) The effect of PGI_2-free myometrial incubate (MI) on the anti-aggregatory activity PGI_2. PGI_2-free incubate was prepared by incubation of myometrial incubate at pH 6.5 at37^{o}C for one hour. Aggregation of citrated rabbit platelet-rich plasma was induced by ADP (5 uM, ▼). MI had no effect on the anti-aggregatory activity of PGI_2 with or without papaverine (lOOum, p) pretreatment.

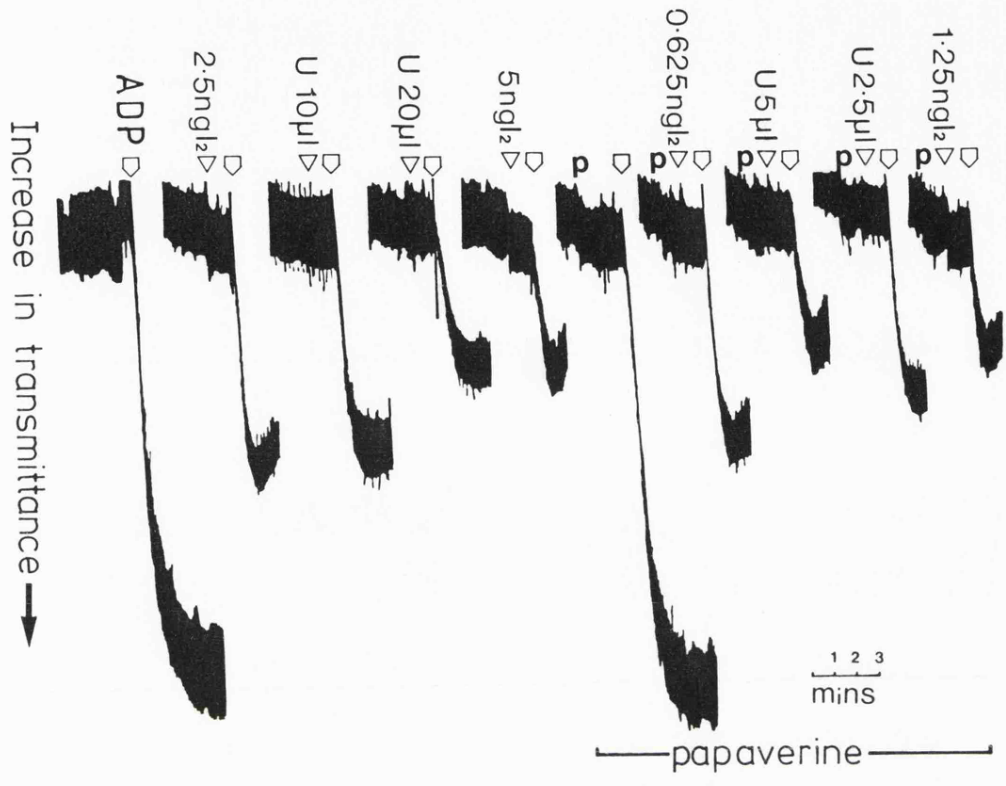


Figure 10) The effect of papaverine (p ; 100uM) on the anti-aggregatory activity of PGI_2 and PGI_2-free human (39-40 week pregnant) myometrial incubate containing 250 ng/ml PGI_2 in a 2+2 doses assay. Papaverine did not affect the response to ADP (10 uM; ▽) but potentiated the effect of PGI_2.

papaverine were 12.6 +/- 3.4 and 9.9 +/- 2.2% respectively. The inter-assay coefficients of variation with and without papaverine were 10.1 +/- 1.6 and 10.3 +/- 1.8 % respectively (see table 2). There were no significant differences between the mean concentrations of PGI_2, or the inter- or intra-assay coefficients of variation using paired 't' test.

4.7) The effect of papaverine on anti-aggregatory activity produced by human myometrium

The potentiation of the anti-aggregatory activity of PGI_2 released by human myometrium by 100uM papaverine was equivalent to that of authentic PGI_2. This enabled the assay of of low quantities of PGI_2 where large quantities of incubation media would be required to inhibit platelet aggregation. Figure 11 illustrates the assay of PGI_2-like anti-aggregatory activity in TBS in which a section of 38 week pregnant myometrium was incubated. 5 and 10 ul of incubation medium exhibited little anti-aggregatory activity and the volumes would have had to be increased to 20 and 40ul to be assayed. The incubation of PRP for 1 minute with 100uM papaverine prior to the addition of the incubation medium potentiated the activity so that the anti-aggregatory activity could be assayed. The amount synthesised by this tissue was 0.654 ng/mg/30 mins.

It was considered that this method provided significant and reliable potentiation of PGI_2 activity which could be used to assay human myometrial PGI_2 released into TBS.

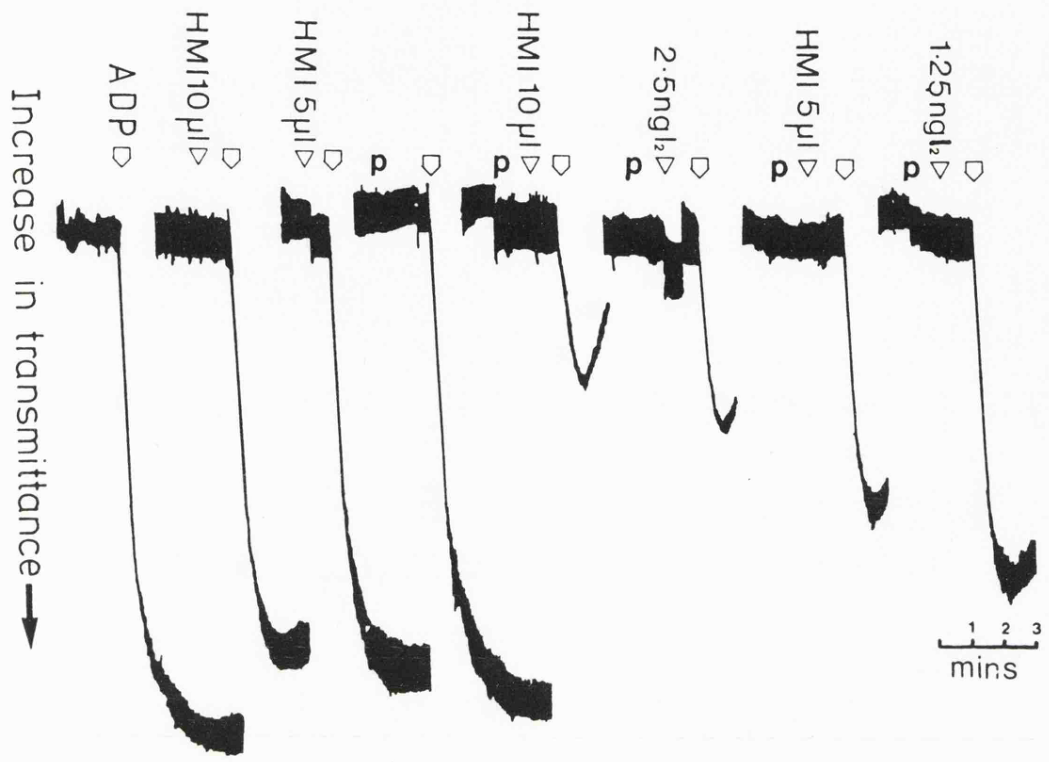


Figure 11) The effect of papaverine (p) on the anti-aggregatory activity present in the incubation medium from human pregnant myometrium(40 week). Aggregation of citrated rabbit platelet-rich plasma was induced by ADP (5uM). Papaverine (p;100 uM) potentiated the activity of the anti-aggregatory activity.

4.8) The effect of citrate concentration on the anti-aggregatory activity of PGI_2

Since the citrate concentration may affect the aggregatory activity of ADP (Orchard, 1982). The effect of altering the concentration on the anti-aggregatory activity of PGI_2 with or without papaverine pre-treatment was assessed . The citrate concentration was reduced from 3.8% to 3.16% (w/v) prior to mixing with rabbit blood (1ml citrate : 9ml blood). The aggregation of PRP (platelet count 700,000) was induced with ADP (10uM) 1 minute after PGI_2 was added. Figure 12 indicates that the anti-aggregatory effect of PGI_2 remained unaltered using PRP prepared with 3.16% citrate when compaired to 3.8% citrate with or without papaverine (100uM).

4.9) Incubation time and the release of placental anti-aggregatory activity

Samples of placenta (delivered vaginally at term) were investigated for the production of anti-aggregatory activity. The release of anti-aggregatory material was studied during incubation in TBS (pH 8.0) after chopping and after pre-incubation at 37°C for 10 minutes prior to chopping. No anti-aggregatory material was released during incubation over a 30 minute period. However significant release was observed after preincubation at 37°C for 10 minutes with subsequent chopping. There was a 32.7% inhibition of ADP-induced platelet aggregation after 10 minutes incubation at room temperature, 34.5% at 20 minutes but inhibitory activity was markedly reduced at 30 minutes to 10.7% and at 45 minutes to 9.5%. For this reason samples were incubated at 37°C prior to chopping and then incubated at room

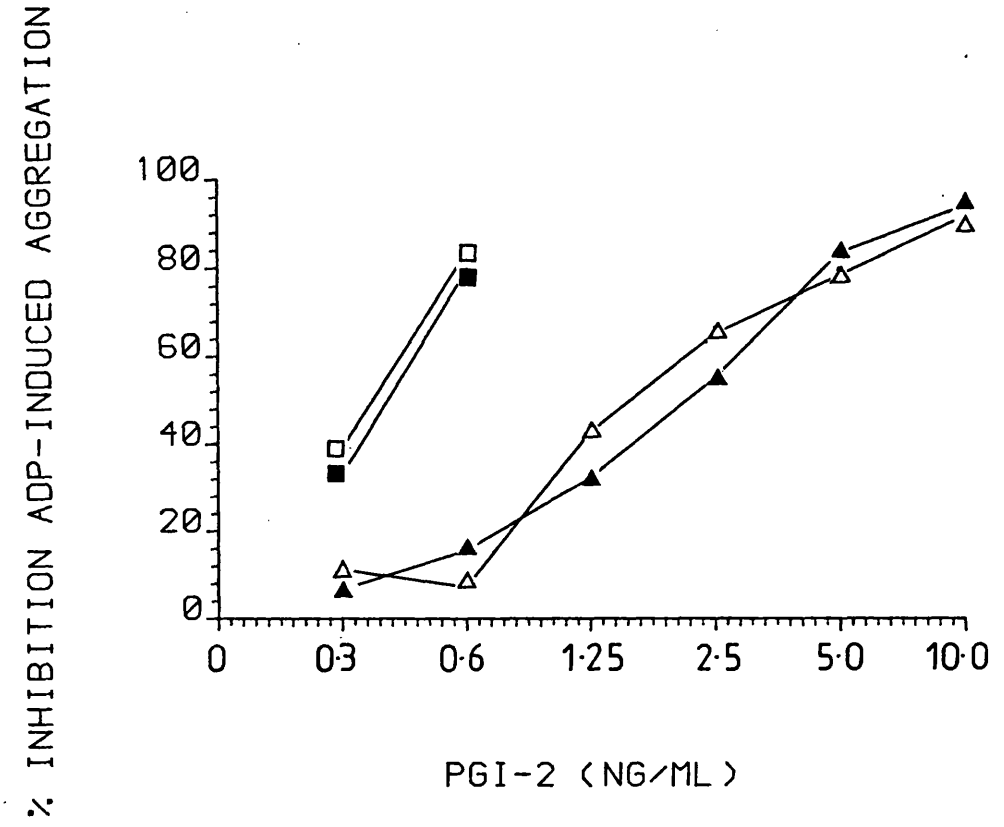


Figure 12) The effect of citrate concentration on the anti-aggregatory activity of PGI_2 in the presence (triangles) and absence of papaverine (100uM, squares). There was no difference seen when PRP was prepared using 3.16% citrate (closed symbols) as opposed to 3.8 % citrate (open symbols).

temperature for 15 minutes. The time elapsed between delivery and assessment of anti-aggregatory activity had no effect on the release of anti-aggregatory material on storage up to 8 hours post-delivery, however all samples were used within 2-4 hours of collection.

4.10) The effect of pH on anti-aggregatory activity released from human myometrium and placenta and rat myometrium

The stability of these activities were tested under severe alkaline conditions. The results from two experiments are summarised in figures 13 and 14. Figure 13 indicates aggregation responses of rabbit PRP induced by ADP (10uM) . Samples of PGI_2, PGD_2, and PGE_2 were tested and alkalinised to pH 12.0 for 60 minutes and reacidified to pH 8.0. The anti-aggregatory activity of PGI_2 (10ng/ml PRP) was identical to that before alkalinisation whilst those of PGD_2 (20 ug/ml) and PGE_2 (60ug/ml) were abolished on alkalinisation.

The alkaline stability of the anti-aggregatory materials released by human and rat pregnant myometrial tissues and human placental tissue was studied in a similar manner (Figure 14). As shown the activities released by the myometrial samples were alkaline stable and similar to PGI_2. However alkalinisation markedly reduced the anti-aggregatory activity derived from the placenta.

It was then decided to compare the stability of the human myometrial and placental activities at lower pH since PGI_2 is unstable at acid pH. As proteins are denatured at low pH, the incubates were incubated at pH 7.5 and 8.5.

The results from one experiment are shown in figure 15. The tracing shows the aggregatory response of rabbit PRP induced by ADP (10uM) . 10ng of authentic PGI_2, 40ul of myometrial incubate and 60ul of

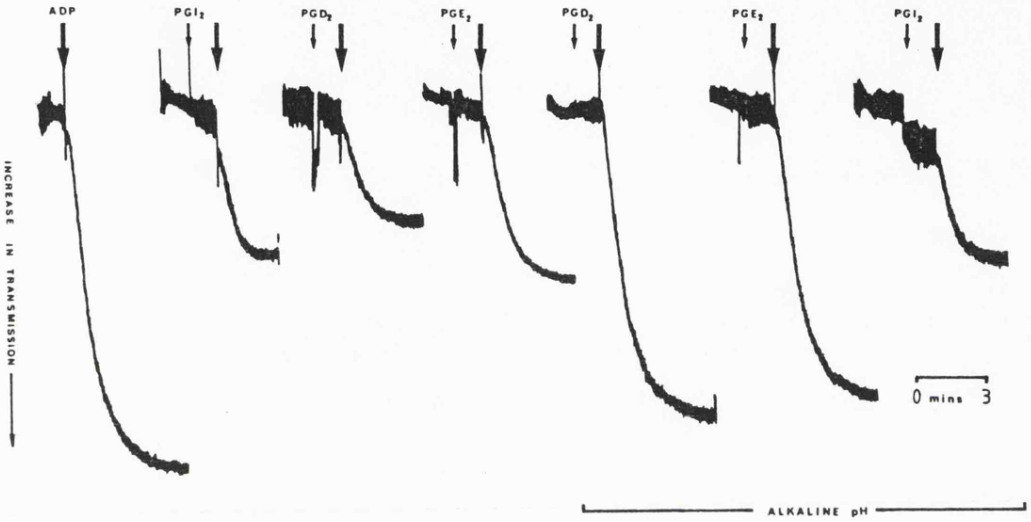


Figure 13) The effect of alkalinisation on the anti-aggregatory activity of authentic PGI_2, PGD_2, and PGE_2. The anti-aggregatory activity of these PGs was tested before and after incubation at pH 12.0 ($4^{o}C$) for 60 minutes. PGI_2 (10ng/ml PRP), PGD_2 (20ug/ml) and PGE_2 (60ug/ml) all exhibited anti-aggregatory activity against ADP(10uM, ↓)-induced aggregation of citrated rabbit PRP. When alkalinised to pH 12.0, PGI_2 retained its activity unlike PGD_2 and PGE_2 which lost all anti-aggregatory activity.

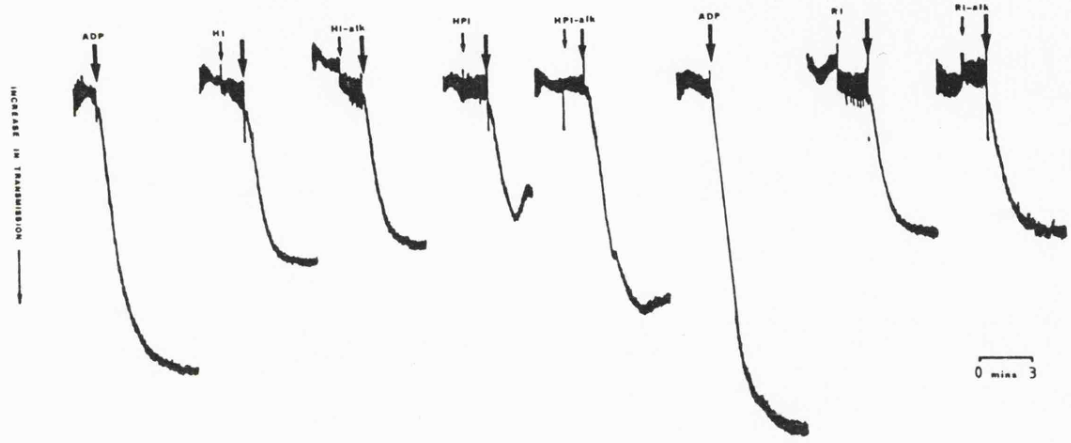


Figure 14) The effect of alkalinisation of pregnant human (HI) and rat (RI) myometrial and human placental (PI) anti-aggregatory activity. All inhibited ADP(10uM, ↓)-induced aggregation of citrated rabbit PRP. Both HI and RI retained anti-aggregatory activity when incubated at pH 12.0 (at 4°C; HI-alk, RI-alk) for 60 minutes, however PI lost some anti-aggregatory activity under these conditions (HPI-alk).

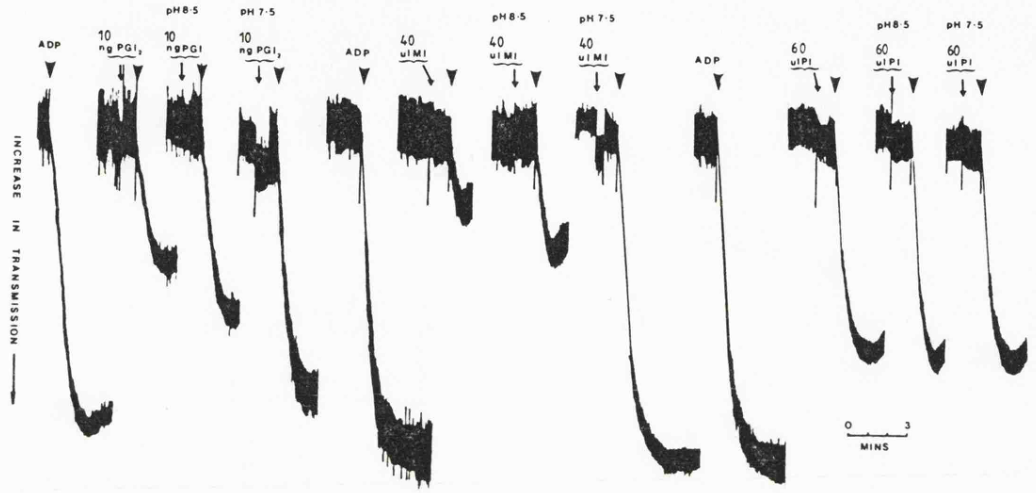


Figure 15) The effect of pH on the anti-aggregatory activities of PGI_2, human pregnant-myometrial (MI) and placental (PI) incubates. The anti-aggregatory activity of PGI_2, MI and PI were assessed using citrated rabbit platelet-rich plasma induced with ADP (10 uM, ▼). The pH was adjusted to pH 8.5 and 7.5 or incubated at room temperature for 1 hour. The anti-aggregatory activity of both PGI_2 and MI at pH 8.5 was slightly reduced but abolished at pH 7.5. The activity of PI was unaffected by these conditions.

placental incubate from a 40 week pregnant woman caused inhibition of the ADP-induced aggregation. Portions of these media were then adjusted to pH7.5 and 8.5 and maintained at room temperature for 30 minutes. The media were then readjusted to pH8.0 and the anti-aggregatory activity tested .

Authentic PGI_2 lost some activity at pH8.5 at room temperature and lost all activity when incubated at pH7.5. The activity released by pregnant myometrium behaved in a similar manner, however the placental factor was unaffected by these incubation conditions .

4.11) The effect of indomethacin on the release of human myometrial and placental anti-aggregatory activity

Human myometrial and placental tissue was incubated with indomethacin (335uM,120 ug/ml) and is illustrated in figure 16. 80ul of placental incubate inhibited ADP (5uM)-induced aggregation and thus 9.6ug of indomethacin was added with the placental incubate to determine any interaction with the anti-aggregatory activity. When 9.6ug of indomethacin was added alone to the PRP there was no effect on ADP induced aggregation. As 40ul of indomethacin treated myometrial incubate was assessed, 4.8ug of indomethacin was added with the myometrial incubate to assess the interaction with the anti-aggregatory activity present.

Indomethacin did not interfere with the anti-aggregatory activity present in myometrial incubate but pre-incubation of the tissue with indomethacin greatly reduced the anti-aggregatory activity present. On the other hand, indomethacin slightly potentiated the anti-aggregatory activity of the placental incubate , but on pre-incubation did not

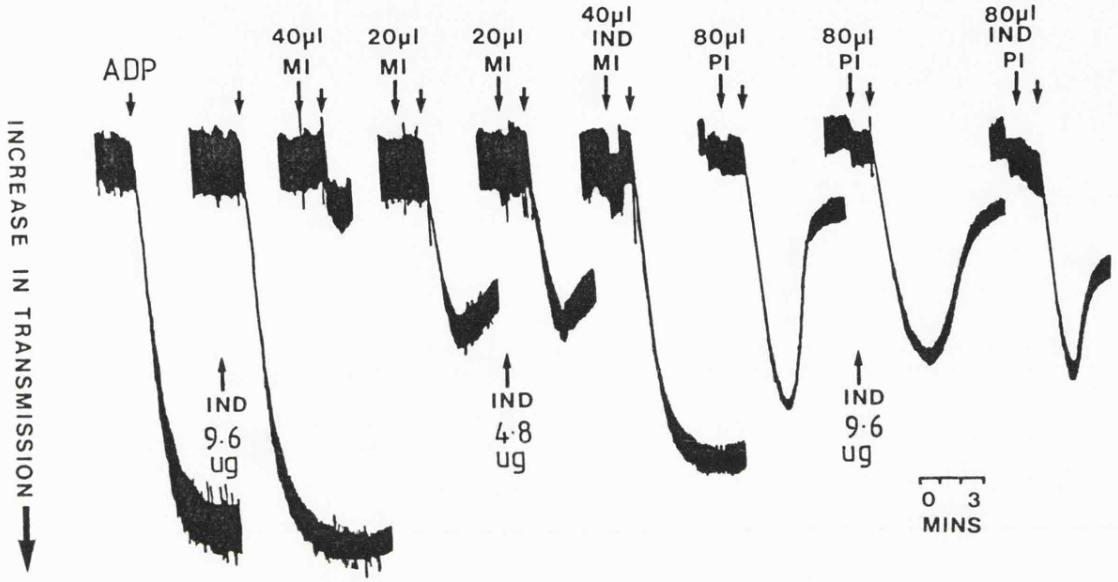


Figure 16) The effect of indomethacin on human pregnant myometrial (MI) and placental (PI) anti-aggregatory activity in incubation media. Platelet aggregation of citrated rabbit platelet-rich plasma was induced by ADP (5 uM; ↓). Addition of indomethacin (4.8-9.6 ug) had no effect on ADP-induced aggregation of the anti-aggregatory activity of MI and PI. Incubation of myometrium with indomethacin (IND MI) reduced the release of anti-aggregatory material whilst not affecting that by the placenta (IND PI).

reduce the release of anti-aggregatory activity.

It was therefore evident that although both the myometrium and placenta release anti-aggregatory material(s), they are not the same. Since the myometrial, activity is stable at alkaline pH, is acid labile, the release inhibited by indomethacin and it's anti-aggregatory activity potentiated by papaverine, the material will be referred to as PGI_2 and the release quantified as PGI_2 equivalents per mg wet weight of tissue per 30 minutes of incubation. PGI_2 is unique among the PGs in possessing all of these properties.

4.12) Assessment of ADPase activity in human myometrial and placental incubation media

Experiments were carried out to determine if the anti-aggregatory factor(s) in the placental and myometrial incubation media were in part due to the presence of an ADPase (Figure 17). To this end, samples of placental and myometrial incubation media were heated at 37°C for 30 minutes to destroy any PGI_2 present and then incubated with ADP (2.3mM) at room temperature for 20 minutes. The aggregatory activity of ADP from stock or incubated solutions were then compared. 5ul of ADP (to make 23uM final PRP concentration) induced irreversible aggregation. 5ul of PGI_2-free myometrial and placental incubates were added with 5ul stock ADP and each had little direct effect on the ADP induced aggregation. If ADP was incubated with the PGI_2-free myometrial incubate, no loss of activity was noted when compared to the response obtained when the incubate was added with stock ADP without incubation. Comparison of the response produced by aliquots of placental incubate compared with the control stock solution showed that 57.0 +/- 1.6% (mean +/- s.e.m.; n=3) of the activity of ADP had disappeared on incubation with the placental

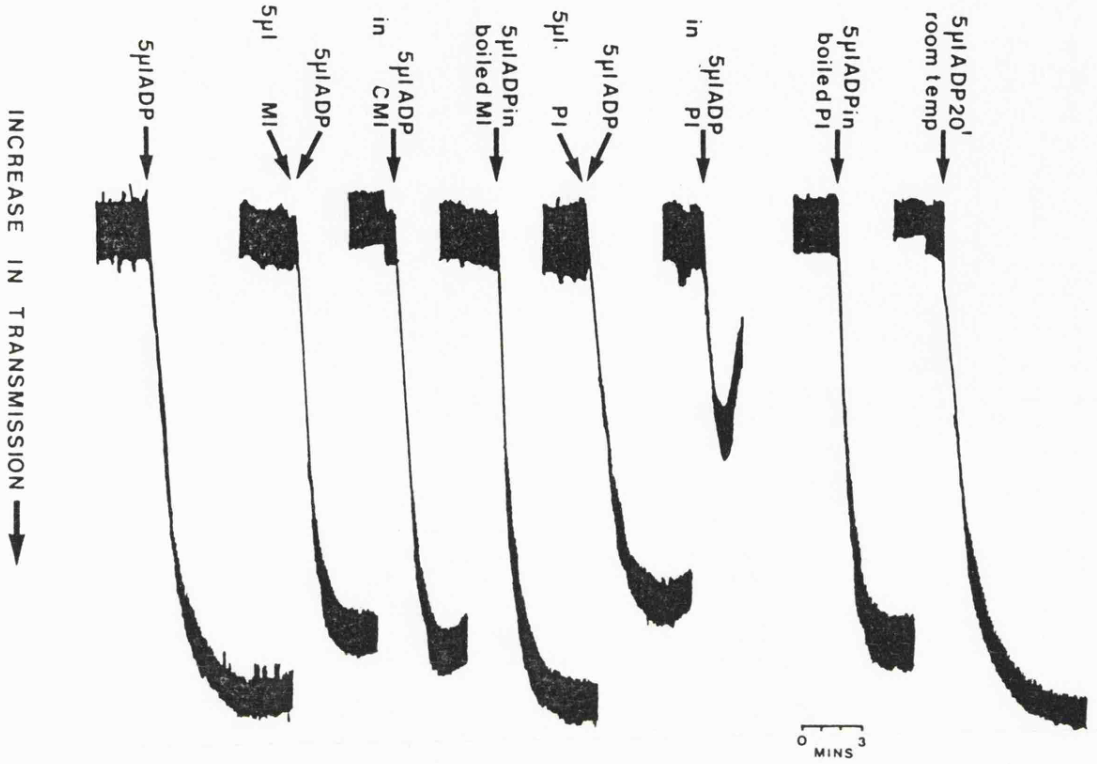


Figure 17) Assessment of ADPase activity in incubation media from human pregnant myometrium and placenta. The incubation media were heated at 37°C to hydrolyse all endogenous PGI_2. ADP (2.3mM) was incubated in PGI_2-free myometrial (MI) and PGI_2-free placental (PI) incubates for 30 minutes at room temperature. The aggregatory activity of the remaining ADP was assessed by inducing aggregation of citrated rabbit platelet-rich plasma. PI had a markedly reduced capacity for inducing aggregation compared to MI and this effect was abolished by boiling. MI and PI had no effect on ADP-induced aggregation when added at the same time. ADP did not degrade over 30 minutes at room temperature.

supernatant. If the supernatant was boiled and centrifuged before incubation with ADP, then no loss of activity was seen.

4.13) The effect of AA on human myometrial PGI_2 synthesis

The effects of two concentrations of AA on human myometrial PGI_2 sythesis is summarised in figure 18. Treatment of myometrial tissue with 50uM AA had no effect on PGI_2 synthesis when compared to the basal synthesis (Basal : 0.16 +/- 0.05 ; AA [50uM] : 0.20 +/- 0.07 ng/mg/30 mins ; n=6) whilst 99uM AA increased synthesis from 0.182 +/- 0.05 to 0.581 +/- 0.27 ng/mg/30 mins ($p<0.05$; n=7). AA carried over in the incubation media did not affect the anti-aggregatory activity of PGI_2.

4.14) The effect of PLA_2 on myometrial PGI_2 synthesis

The effects of two concentrations of PLA_2 on human myometrial PGI_2 synthesis is shown in figure 19. Treatment of the tissues with PLA_2 (10U/ml) increased generation from 0.17 +/- 0.07 to 0.48 +/- 0.2 ng/mg/30 mins ($p<0.02$; n=5). PLA_2 at a concentration of 5U/ml did not affect PGI_2 synthesis (Basal : 0.160 +/- 0.05 ; PLA_2 [5U/ml] : 0.12 +/- 0.04 ng/mg/30 mins ; n=6).

4.15) The effect of oxytocin and ergometrine on pregnant human myometrial PGI_2 synthesis

The effect of oxytocin (0.44uM) and ergometrine (61uM) on myometrial PGI_2 synthesis is summarised in figure 20. Incubation of human myometrium with oxytocin or ergometrine did not alter PGI_2 synthesis. The basal synthesis of 0.264 +/- 0.09 was increased to 0.317 +/- 0.17 ng/mg/30mins (n=8) in the presence of oxytocin . Ergometrine

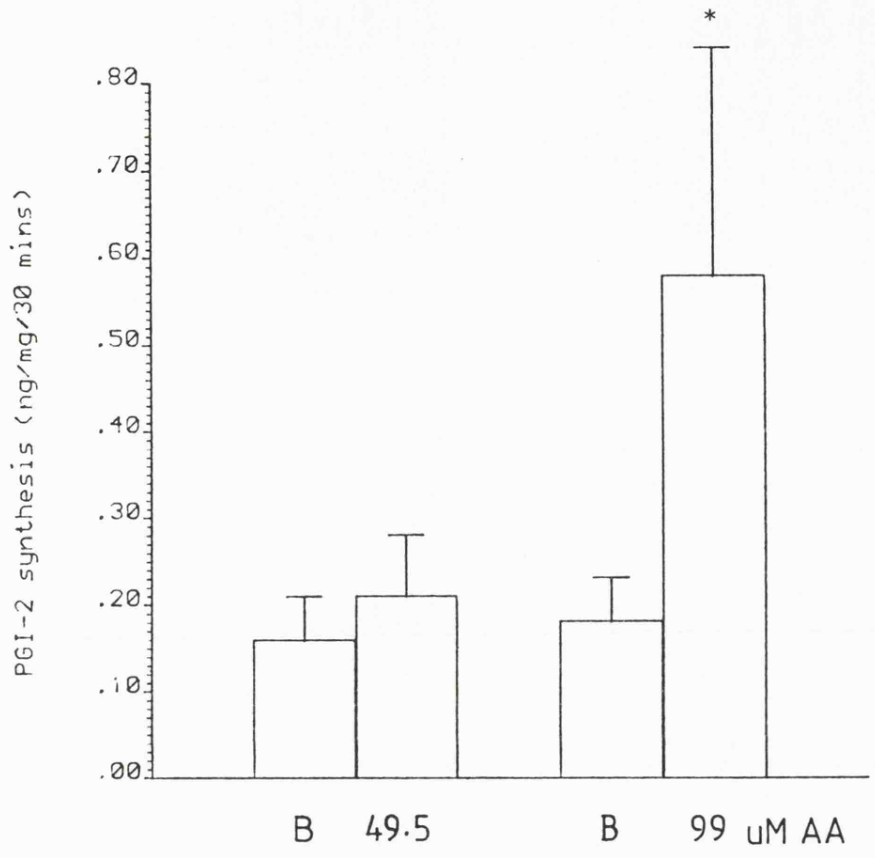


Figure 18) The effect of arachidonic acid (AA) on the production of anti-aggregatory activity by human pregnant myometrium (36-39 week, B). Inhibition of ADP-induced aggregation of citrated rabbit platelet-rich plasma by M was not increased by 49.5 uM AA (n=6) but was increased in the presence of 99.0 uM AA (n=7). Columns and vertical lines represent mean +/- s.e.m. Significant differences between control and treated groups are shown (* $p<0.02$).

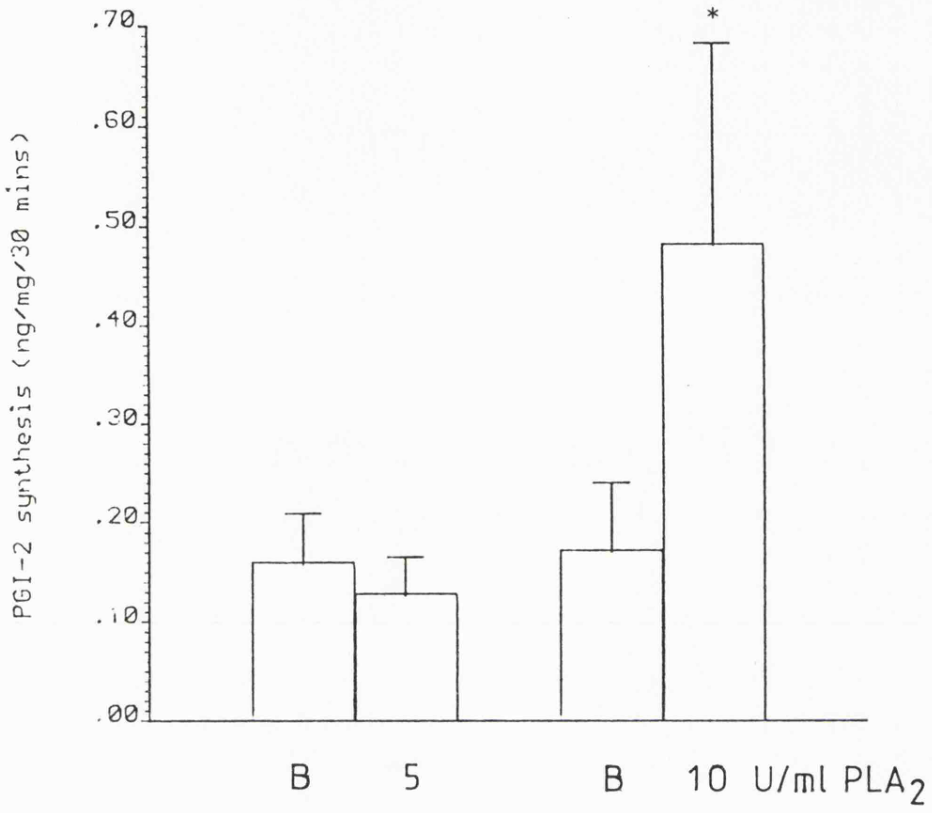


Figure 19) The effect of phospholipase-A_2 (PLA_2) on the formation of human pregnant (36-39 weeks gestation) myometrial anti-aggregatory activity (M) in the incubation medium. Inhibition of ADP-induced aggregation of citrated rabbit platelet-rich plasma by M was not increased by 1 U/ml PLA_2 (n=6) but was increased in the presence of 10 U/ml PLA_2 (n=5). Columns and vertical lines represent mean +/- s.e.m. Significant differences between basal (B) and treated groups are shown (* $p<0.02$).

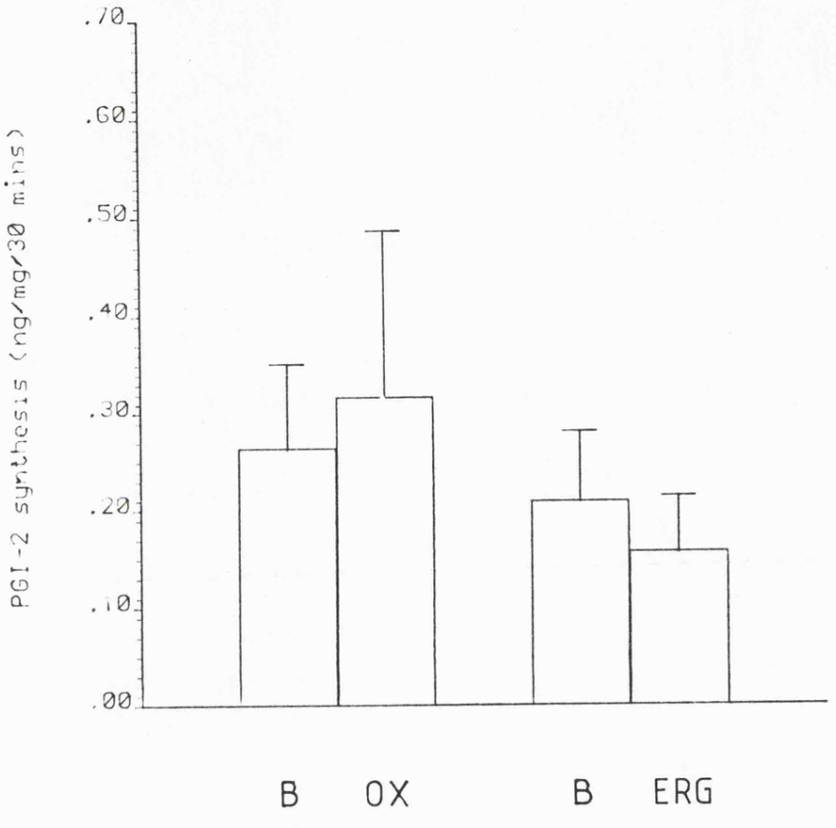


Figure 20) The effect of oxytocin (OX) and ergometrine (ERG) on human pregnant myometrial PGI_2 synthesis (38-40 weeks gestation). Basal synthesis (B) of PGI_2 was not significantly influenced by oxytocin (0.44 uM, n=8) or ergometrine (6luM n=5). Columns and vertical bars indicate mean +/- s.e.m.

treatment decreased PGI_2 production from 0.209 +/- 0.07 to 0.157 +/- 0.06 ng/mg/30 mins (mean +/- s.e.m.; n=5).

4.16) Prostacyclin production by human myometrium during pregnancy

The variation in PGI_2 synthesis that occurs in myometrial tissue during gestation is shown in figure 21. Synthesis in the second trimester (week 15) of pregnancy was low (0.093 +/- 0.038 ng/mg/30 mins; n=3) and synthesis increased two weeks prior to term. Synthesis at 37 weeks was 0.939 ng/mg/30 minutes (n=2), week 38 : 0.129 +/- 0.018 (n=8), week 39 : 0.262 +/- 0.058 (n=10) peaking at term (week 40) ; 0.473 +/- 0.0826 (n=9). PGI_2 synthesis from two patients who had not started labour by week 42 had low syntheses at 0.216 and 0.0343 ng/mg/30 minutes repectively.

4.17) The effect of PGI_2 on human lower segment uterus in vitro

Four myometrial strips (weeks 38 - 40) were suspended under a 1g load in 10ml Krebs' solution gassed with 5% CO_2 : 95% O_2. After 1 hour equilibration all tissues responded to 200ng/ml Ach and exhibited isotonic contractions. After 1 hour equilibration all tissues exhibited isotonic contractions. 200 ng/ml PGI_2 inhibited spontaneous activity in three samples and reduced the basal tone in two (see figure 22). Lower concentrations (20 and 2 ng/ml) did not affect activity in two other preparations. Spontaneity did not resume after washing.

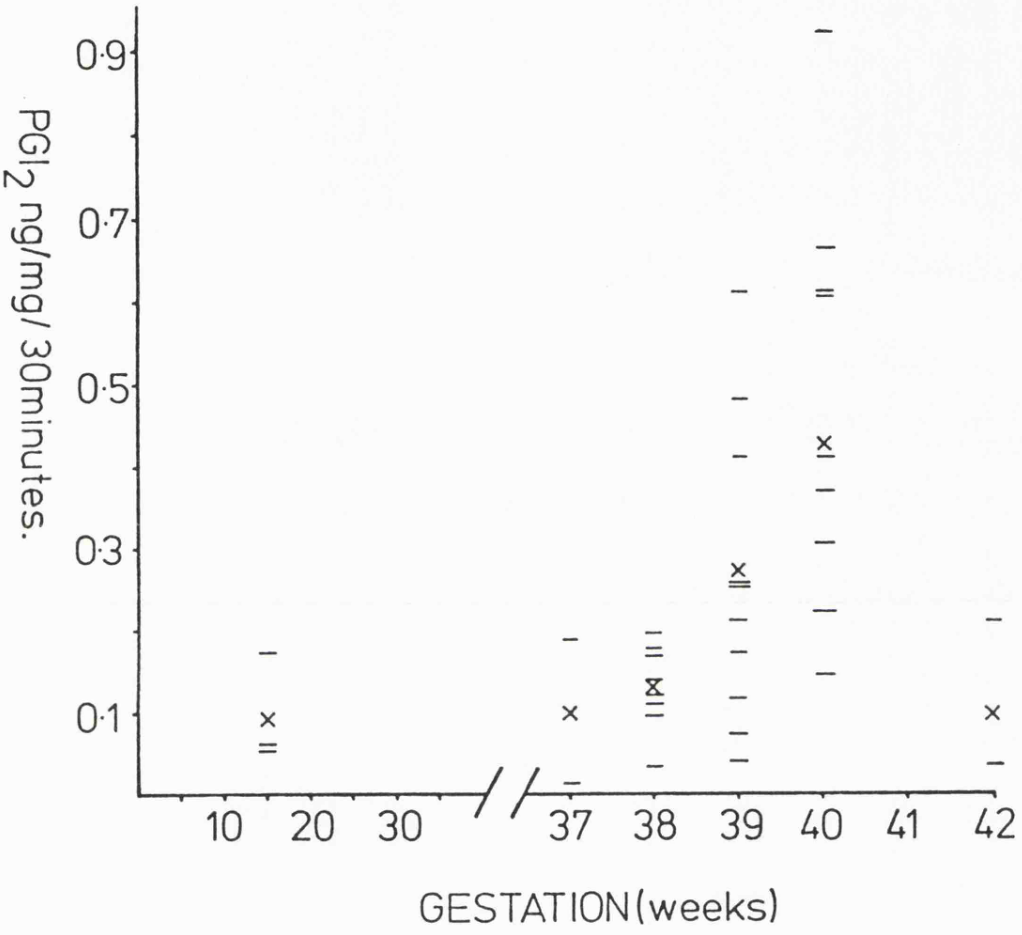


Figure 21) The effect of gestation on human myometrial PGI_2 synthesis. Synthesis was maximal at term. Production by myometria from patients of 42 weeks gestation (not in labour) was at a level similar 1st trimester patients. (X) indicates mean and (-) individual values.

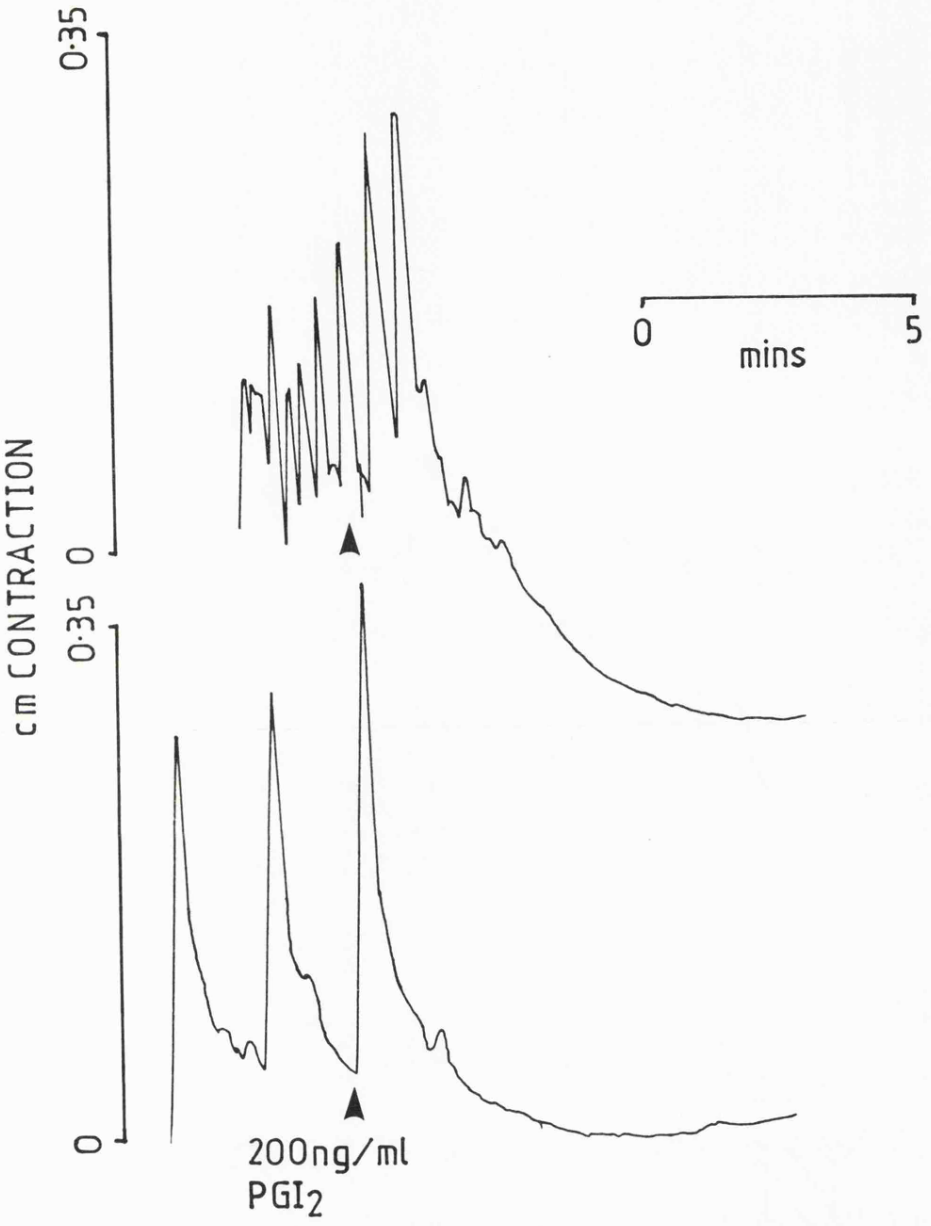


Figure 22) The effect of PGI_2 (200 ng/ml) on 2 pieces of human lower segment 40 week pregnant myometrium . The tissues were bathed in Krebs' solution at $37^{o}C$. Spontaneous contractions were abolished in each case and basal tone reduced.

5.0) RESULTS - Studies on the production of PGI_2 by the pregnant rat myometrium

5.1) The effect of tissue handling on PGI_2 synthesis by rat pregnant myometrium

Rat pregnant myometria from 5 animals were split into 4 pieces and each was treated differently. The decidual tissue of 2 pieces was separated by scraping with a microscope slide and in the other two pieces by gentle teasing with a cotton wool bud. All samples were pre-incubated at 37°C for 10 minutes. One sample from each group was then chopped prior to incubation at room temperature. The results are illustrated in figure 23.

Scraped tissue synthesised greater amounts of PGI_2 than teased tissue ($p<0.05$; ANOVA at 20 minutes). Chopping increased the rate of PGI_2 synthesis by the scraped tissue reaching a maximum at 20 minutes compared to 40 minutes without chopping . The PGI_2 content of the scraped and chopped media began to fall after 28 minutes.

5.2) The effect of oxytocic drugs on rat myometrial PGI_2 synthesis

5.2 i) 5-HT

The effect of incubating myometrial tissue with 5-HT (21uM) on PGI_2 synthesis is illustrated in figure 24. Basal PGI_2 synthesis was assayed using a 2+2 doses assay against authentic PGI_2. However 5-HT may affect the aggregability of the platelets and thus controls for the carry-over of 5-HT were carried out. The maximum volume of the incubate from the day 19 pregnant myometrial incubation mixture containing 5-HT used in the assay was 5ul. This is a carry-over of 0.1 ug 5-HT (21uM , 20ug/ml) and this dose was added to PRP and its effect on ADP induced aggregation alone was examined. This amount was also added with the authentic PGI_2 when compared with the test sample. 2.5ul 5-HT incubate

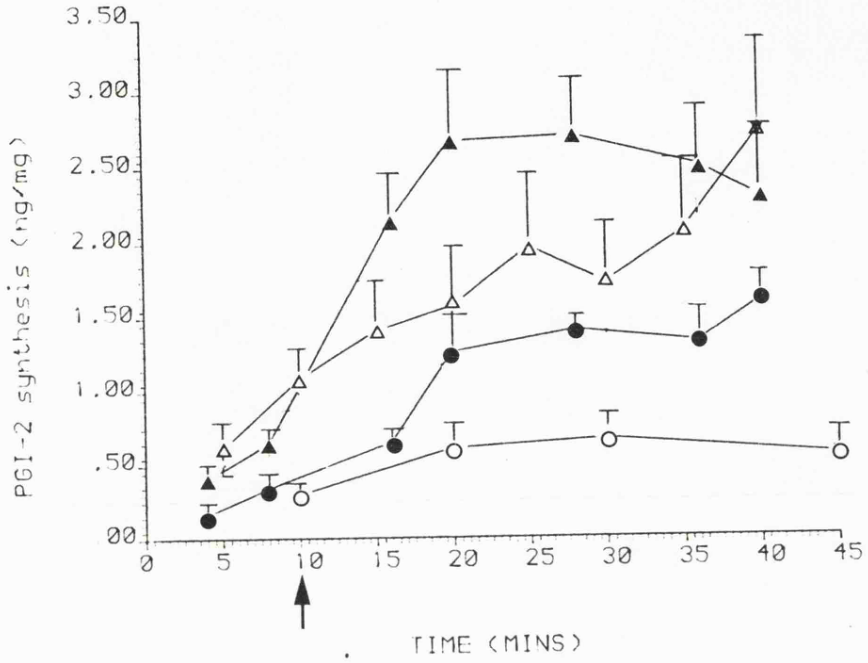


Figure 23) The effect of handling of rat uterine tissue (19-20 day pregnant) during separation of myometrium from deciduum on PGI_2 formation. Decidual tissue was removed by scraping (triangles) or teasing (circles). The two groups were then either chopped (closed symbols) after 10 minutes preincubation at 37°C or left intact (open symbols). Scraping increased PGI_2 synthesis over teasing, and chopping increased the synthesis further. (↑) indicates chopping where applicable.

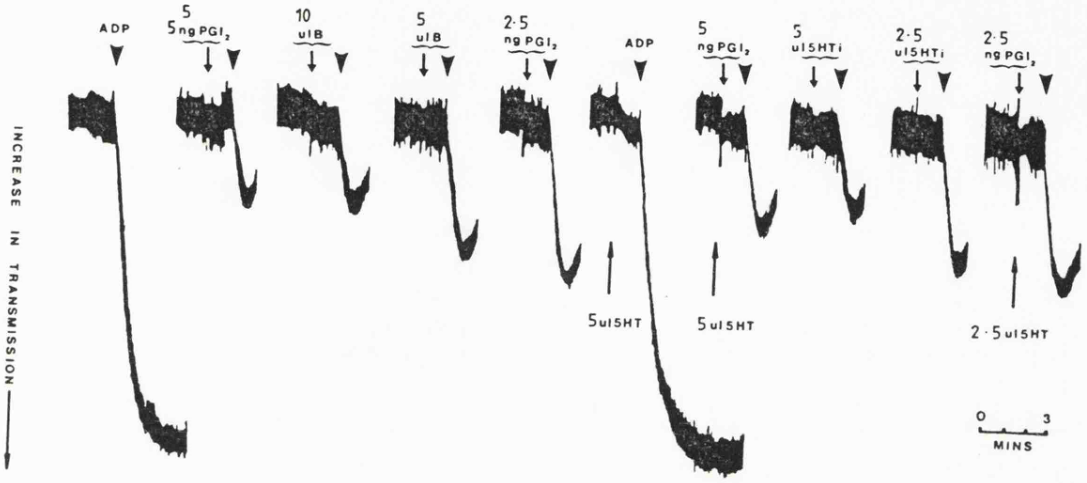


Figure 24) The effect of 5-HT (21uM) on PGI_2 synthesis by rat pregnant (day 19; B) myometrium. Addition of 100ng of 5-HT in 5ul Tris-buffered saline (50 mM,pH 8.0) had no effect on ADP-induced aggregation of citrated rabbit platelet-rich plasma nor on the anti-aggregatory activity of PGI_2. PGI_2 synthesis in the presence of 5-HT (5HTi) was markedly stimulated from 1.625 to 3.732 ng/mg/15 mins.

would contain 0.05ug 5-HT and this was added to the authentic PGI_2 to control for the lower dose of incubate.

5-HT at these doses had no effect on ADP-induced aggregation nor upon the anti-aggregatory activity of PGI_2 . 5-HT more than doubled the PGI_2 synthesis from 1.625 ng/mg/15 mins to 3.732 ng/mg/15 mins, necessitating a reduction in the volume of 5-HT treated incubate added to the platelets from 10ul and 5ul to 5ul and 2.5ul.

5-HT increased basal PGI_2 synthesis in a day 19 pregnant myometrium in a dose-related manner (figure 25). Basal synthesis of 2.449ng/mg/15 mins was increased to 2.891 (+18.1%) by 5.3uM 5-HT; to 4.00 (+63.3%) by 10.5uM ; to 4.639 (+89.4%) by 21uM and to 5.59 ng (+128.3%) by 84uM 5-HT.

This graded increase suggested a receptor mediated effect and in another experiment rat myometrial tissue was incubated with 5-HT with or without methysergide (17.6uM : see figure 26). Tissue was pre-incubated with methysergide for 10 minutes prior to the addition of 5-HT and then pre-incubated for a further 10 minutes before chopping. Controls during the assays were instituted as illustrated in figure 24 , and those samples were assayed against authentic PGI_2 incubated with the appropriate drug mixtures.

Five pregnant rats (day 20) were used. 5-HT (42uM) stimulated basal synthesis from 3.167 +/- 0.45 to 4.289 +/- 0.49 ng/mg/15 mins ($p<0.05$). Methysergide reduced basal synthesis to 2.191 +/- 0.29 ng/mg/15 mins and no stimulation of PGI_2 synthesis was seen when myometrium was pre-incubated with 5-HT in the presence of methysergide.

5.2 ii) Angiotensin and oxytocin

A day 19 pregnant rat myometrium (figure 27) released 2.011

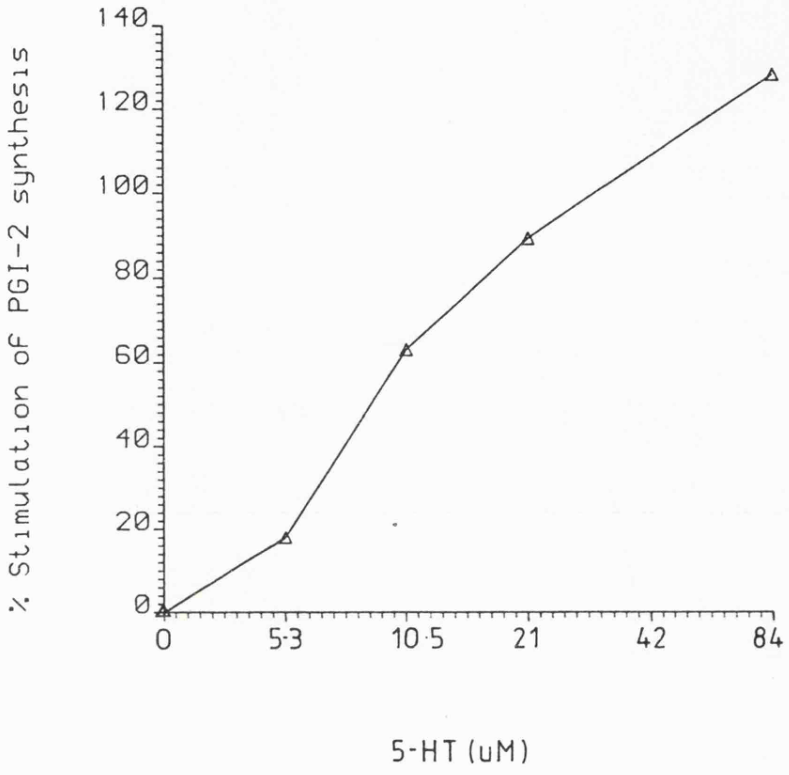


Figure 25) The effect of 5-HT on PGI_2 synthesis by the myometrium of a day 19 pregnant rat. 5-HT stimulated myometrial synthesis in a dose dependant fashion.

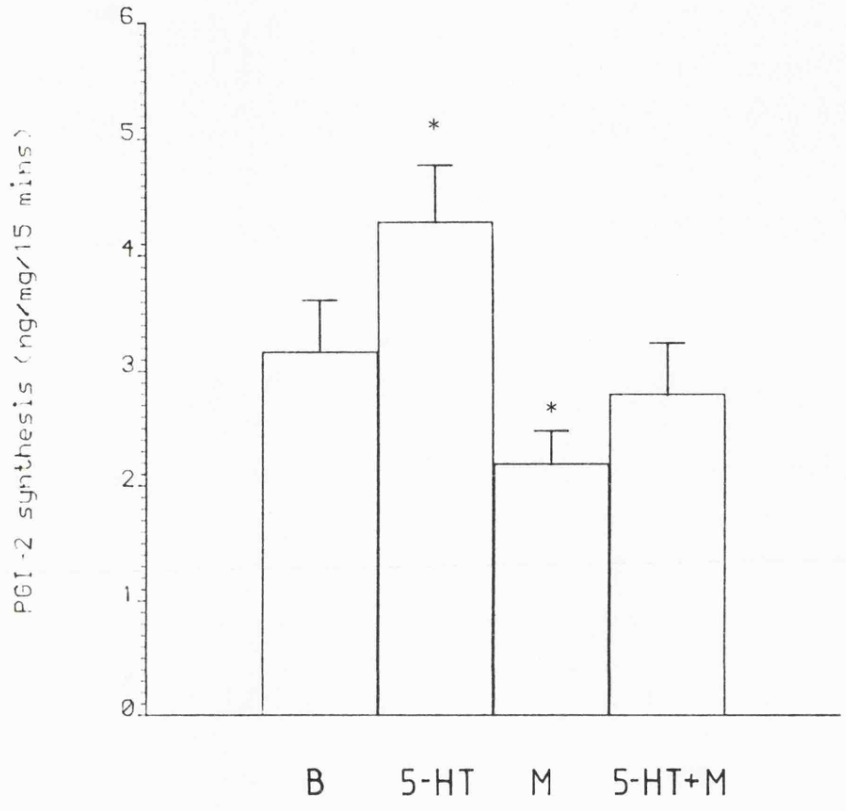


Figure 26) The effect of 5-HT on rat myometrial PGI_2 production (19-20 day pregnant) and its blockade by methysergide (M). 5-HT (42uM) stimulated basal (B) PGI_2 synthesis which was antagonised by methysergide (17.6uM). Columns and vertical lines indicate mean +/- s.e.m. Significant differences are indicated (* $p<0.05$, n=4).

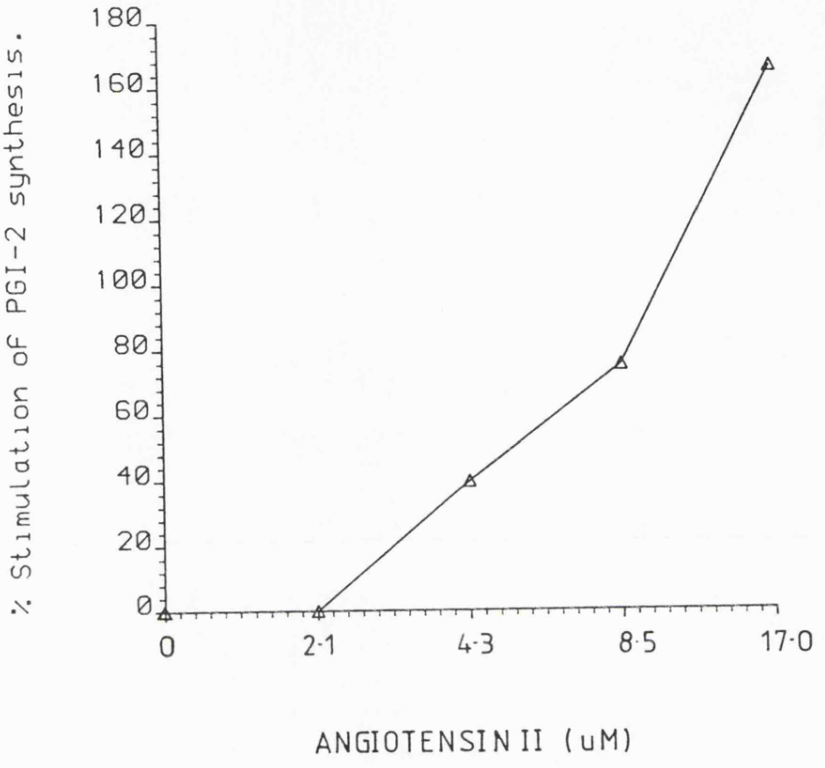


Figure 27) The effect of angiotensin II on PGI_2 synthesis by myometrium from a day 19 pregnant rat. Angiotensin II stimulated synthesis in a dose-related manner.

ng/mg/15 mins. This was stimulated in a dose related manner by angiotensin II to 2.028 (+0.82%) at 0.63ug/ml;to 2.812 (+39.83%) at 1.25ug/ml; to 3.54 (+75.7%) by 2.5ug/ml and to 5.341 ng/mg/15 mins (+165.6%) by 5ug/ml.

Oxytocin also stimulated day 18 rat myometrial synthesis in a dose related manner (Figure 28) from 1.648 (basal release) to 1.837 (+11.6%) at 0.11uM; to 2.197 (+33.4%) at)0.22uM; to 3.389 (+105.8%) at 0.44uM and to 4.17 ng/mg/15 mins (+153.0%) at 0.88uM.

Controls were carried out for both oxytocin and angiotensin in the same manner as those used for 5-HT to eliminate carry-over effects on the platelets.

5.3) The effect of salbutamol on uterine PGI_2 synthesis

The incubation of myometrial tissue with salbutamol (21.5uM) resulted in an inhibition of PGI_2 synthesis (Figure 29). 5ul and 2.5ul of incubate were used for bioassay. These volumes, if taken from an incubation mixture containing salbutamol will carry-over 0.1 ug and 0.05ug respectively. As the tracing shows, 5ul of TBS containing 0.1ug salbutamol did not affect ADP-induced aggregation or the anti-aggregatory activity of PGI_2. Salbutamol reduced synthesis from 4.0 to 2.37 ng/mg/15 mins.

The effect of salbutamol on myometrial PGI_2 production varied on different days of pregnancy (Figure 30). Salbutamol (43uM) only inhibited PGI_2 synthesis in late pregnant rats. On day 21 ; basal PGI_2 production of 4.414 +/- 0.831 was reduced by salbutamol to 1.994 +/- 0.31 ng/mg/15 mins ($p<0.05$; n=5). However in 19 day pregnant myometria, where basal PGI_2 synthesis was 2.548 +/- 0.13 , salbutamol had little effect (2.40 +/-0.35) ng/mg/15 mins (n=4). Salbutamol reduced the PGI_2

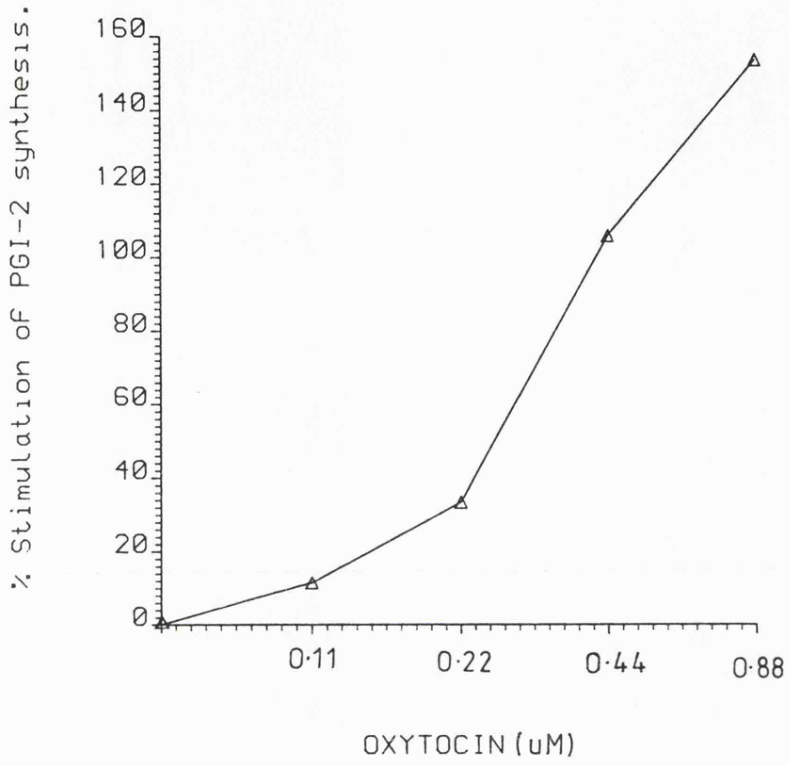


Figure 28) The effect of oxytocin on PGI_2 synthesis by myometrium from a day 20 pregnant rat. Oxytocin stimulated PGI_2 synthesis in a dose related manner.

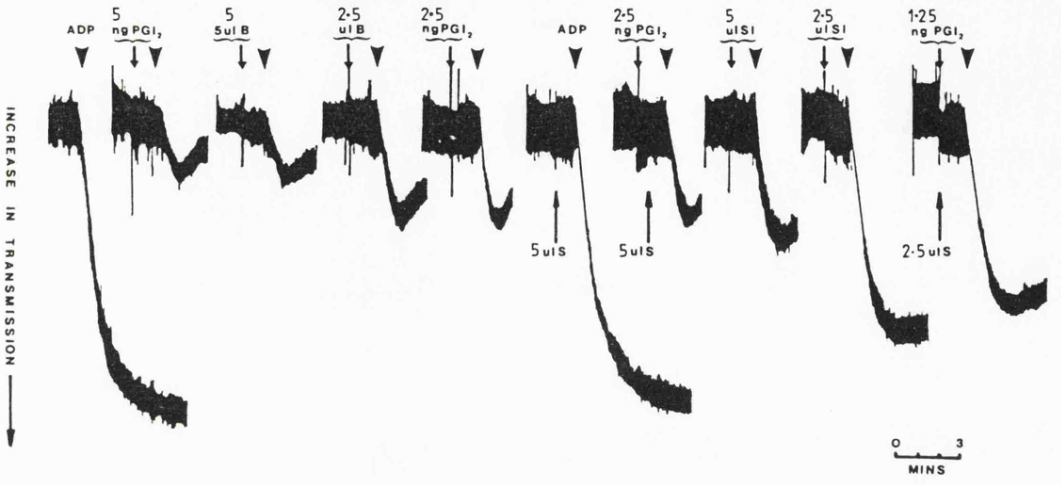


Figure 29) The effect of salbutamol (21.5 uM, SI) on basal (B) PGI_2 synthesis by rat pregnant (day 21) myometrial tissue. Addition of 100ng of salbutamol (S) in 5ul of Tris buffered (50mM,pH8.0) saline had no effect on ADP(10uM, ▼)-induced aggregation of citrated rabbit platelet-rich plasma nor the anti-aggregatory action of PGI_2. PGI_2 synthesis in the presence of salbutamol was reduced from 4.00 to 2.370 ng/mg/15 mins.

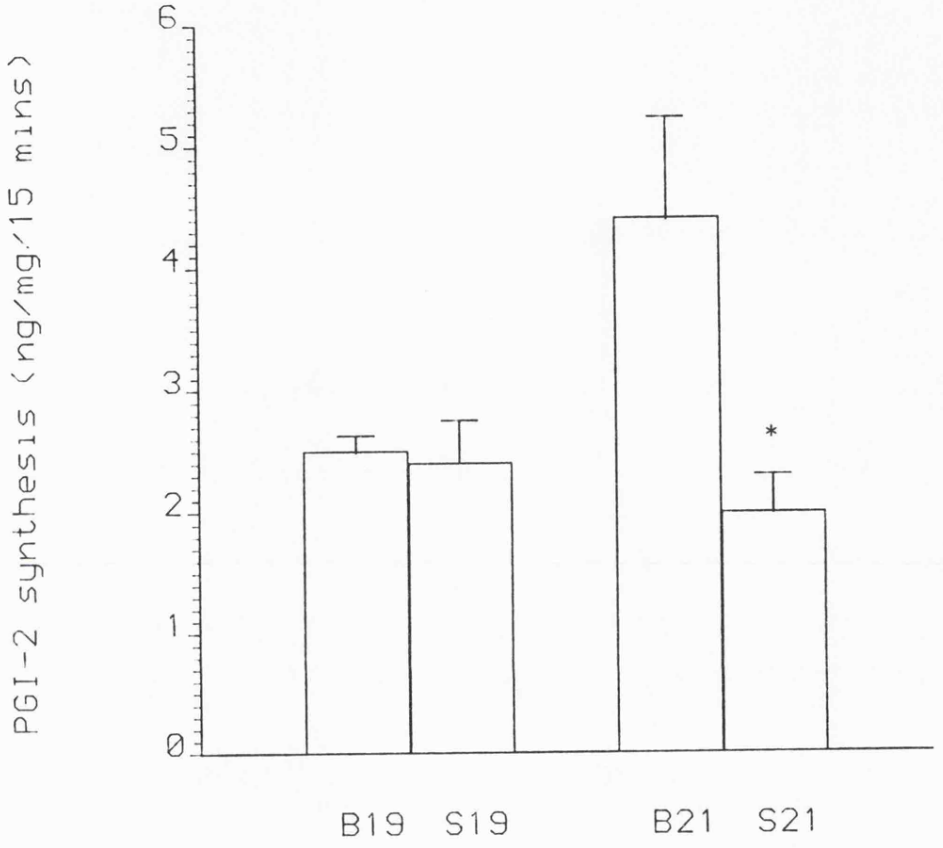


Figure 30) The effects of salbutamol on rat myometrial PGI_2 synthesis. PGI_2 synthesis by day 21 (B21, n=5) pregnant myometrium is markedly higher than day 19 (B19, n=4). Salbutamol (43 uM) inhibits PGI_2 synthesis by 21 day pregnant myometrium (S21) but not 19 day pregnant myometrium. Significant differences between the basal and treated groups is indicated (* $p<0.05$).

release of the day 21 pregnant myometrium to that seen at day 19.

Pretreatment of rat myometrium with propranolol (figure 31) reduced PGI_2 synthesis from 4.414 +/- 0.831 to 3.426 +/- 0.85 ng/mg/15 mins (n=5). This decrease was not significant. However in the presence of propranolol salbutamol did not reduce PGI_2 formation (3.234 +/- 0.66 ng/mg/15 mins).

5.4) The effect of vasodilators on rat myometrial PGI_2 synthesis

5.4 i) Dipyridamole

Day 19-20 pregnant myometria were treated with dipyridamole and the effect on PGI_2 production is shown in figure 32. Dipyridamole required dilution in ethanol to ensure solubility in TBS. Incubation of myometrial samples in ethanol was thus required to ensure a valid comparison of basal synthesis with that of dipyridamole. All ethanol-containing incubation media contained significantly less PGI_2 than basal controls ($p<0.05$, Dunnets test). Basal release of 2.357 +/- 0.52 ng/mg/15 mins (n=4) was reduced to 1.165 +/- 0.41 by 7.4% ethanol which was stimulated in turn to 1.26 +/- 0.34 by 0.1 mM dipyridamole. 14.7% ethanol reduced basal synthesis to 1.594 +/- 0.22 (n=7) which was stimulated to 3.767 +/- 0.69 by 0.2 mM dipyridamole ($p<0.05$, paired 't' test) and the reduced PGI_2 synthesis in the presence of 29.4% ethanol was stimulated from 1.52 +/- 3.34 (n=4) to 2.791 +/- 1.1 ng/mg/15 mins by 0.4mM dipyridamole.

The appropriate doses of dipyridamole and ethanol were added with the authentic PGI_2 standards to account for the carry over. The carry over from the samples containing 0.4mM dipyridamole potentiated the anti-aggregatory activity of endogenous and authentic PGI_2.

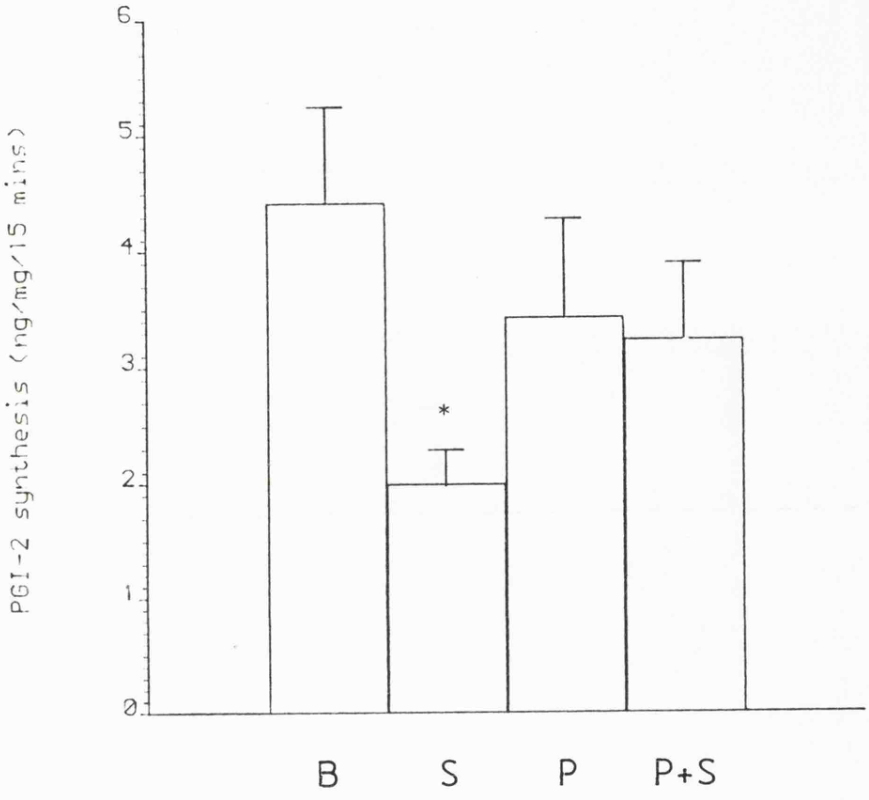


Figure 31) The effect of propranolol (P) on basal (B) and salbutamol (S) inhibited synthesis of rat myometrial PGI_2 (day 21 pregnancy, n=5). Synthesis of PGI_2 was reduced by salbutamol (43 uM) and propranolol (38.6 uM). S did not inhibit PGI_2 synthesis in the presence of propranolol. Columns and vertical lines indicate means +/- s.e.m. Significant differences between basal and treated groups are indicated (* $p<0.05$).

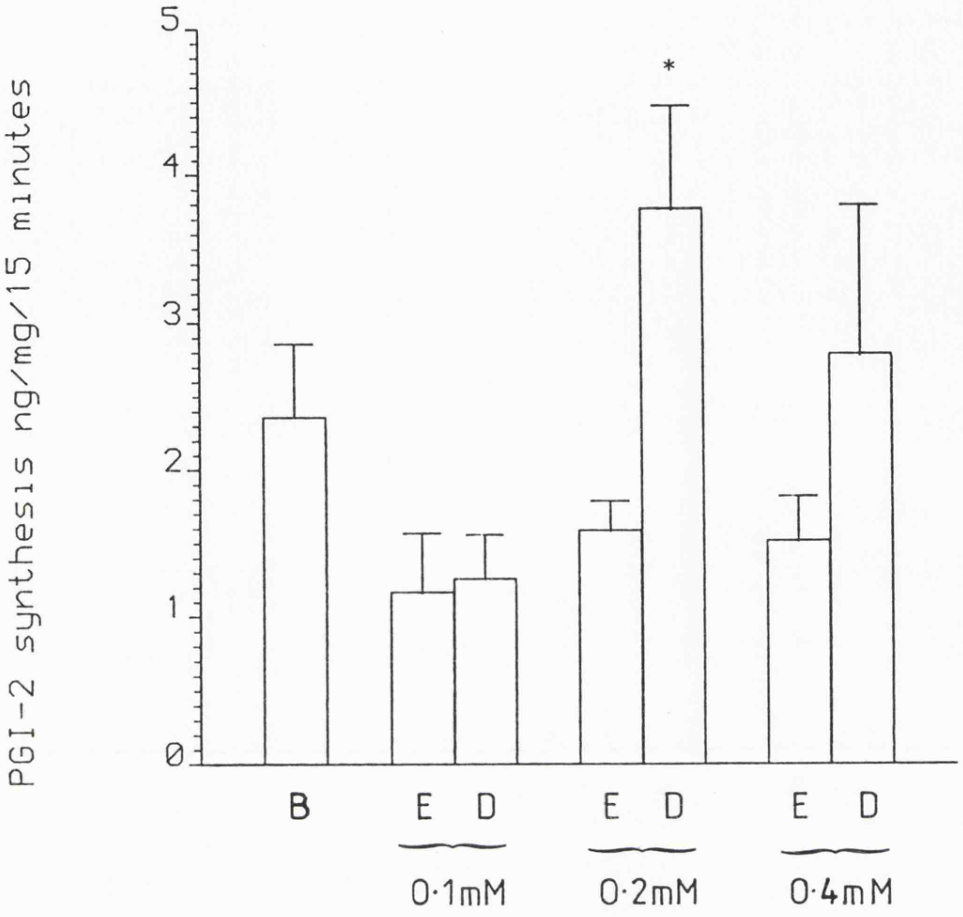


Figure 32) The effect of dipyridamole (DP) on rat myometrial PGI_2 synthesis (day 19-20 pregnancy). Basal (B) synthesis was reduced by ethanol vehicle (E) in all treated groups. DP (0.2 & 0.4 mM) stimulated synthesis (n=7 and n=4 respectiv ely) over ethanol controls whilst 0.1mM DP did not. Columns and vertical lines indicate mean +/- s.e.m. Significant difference between basal vehicle controls and treated groups are shown (* $p<0.05$).

5.4 ii) Hydralazine

Hydralazine when incubated with 20 day pregnant rat myometrial tissue (n=4), reduced PGI_2 synthesis significantly (Figure 33). Basal synthesis was reduced from 1.48 +/- 0.11 ng/mg/15 mins to 0.59 +/- 0.13 ng/mg/15 min by 1 mM hydralazine ($P<0.05$, Dunnets test) to 0.2 +/- 0.05 in the presence of 2mM ($p<0.05$) and to 0.1 +/- 0.04 by 4mM ($p<0.01$). The appropriate doses of hydralazine were added with authentic PGI_2 standards to account for the carry-over of hydralazine. Hydralazine did not affect the anti-aggregatory activity of PGI_2.

5.5) The effect of Forskolin on rat myometrial PGI_2 synthesis.

Day 21 pregnant rat myometria were pre-incubated with Forskolin for 10 minutes prior to chopping and assay for PGI_2. Forskolin potentiated the anti-aggregatory activity of PGI_2 and thus incubates from treated myometrial tissue were assayed against authentic PGI_2 added with the equivalent amount of forskolin. Figure 34 shows that forskolin (25uM) inhibited basal PGI_2 synthesis from 3.47 +/- 0.51 ng/mg/15 minutes to 3.29 +/- 0.35 (N.S.); 50uM reduced production to 2.49 +/- 0.22 ($p<0.05$) and 100uM to 1.435 +/- 0.35 ng/mg/15 minutes ($p<0.02$).

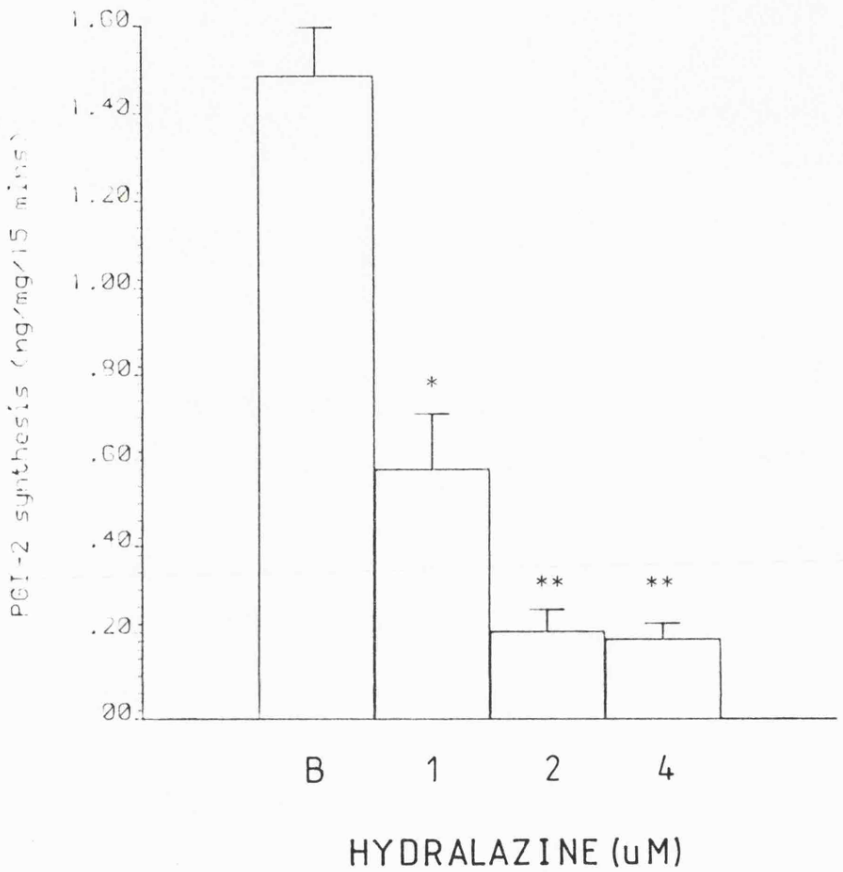


Figure 33) The effect of hydralazine on rat myometrial PGI_2 synthesis (day 20 pregnancy). Basal (B) synthesis of PGI_2 was inhibited in the presence of increasing doses of hydralazine. Columns and vertical lines represent mean +/- s.e.m. Significant differences between basal and treated groups are shown (** $p<0.01$).

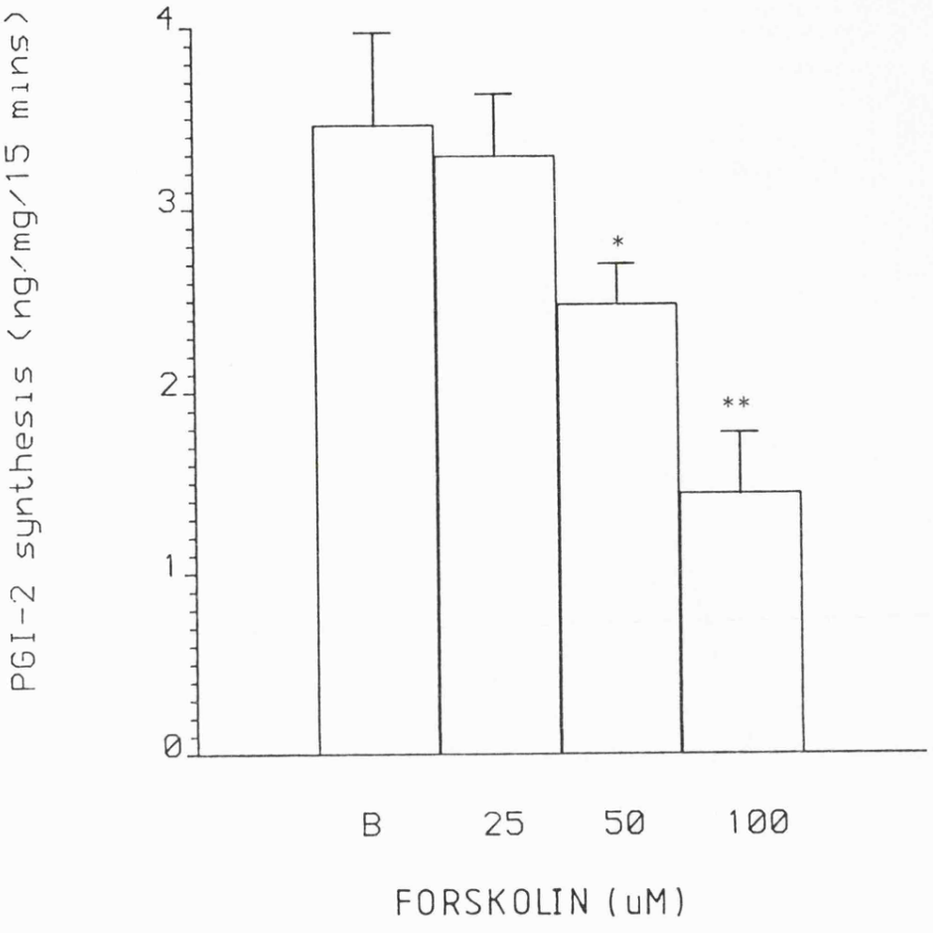


Figure 34) The effect of forskolin on rat myometrial (21 day pregnant) PGI_2 synthesis. Forskolin reduced synthesis in a dose-related fashion. Vertical bars indicate mean +/- s.e.m. Significant differences from basal release are indicated (* $p<0.05$; ** $p<0.02$).

6.0) RESULTS - Assay of pregnant rat uterine PLA_2 activity

6.1) Characterisation of the procedure for the assay of rat uterine phospholipase-A_2

Three incubation media (see section 3.4) were assessed for their suitability for the assay of PLA_2 taking into account their behaviour in the extraction and separative processes used. dPC and oleic acid were separated using either mini-columns or thin-layer chromatography. Extraction was carried out with either mini-columns or, prior to separation by TLC, the method of Bligh and Dyer (1959).

6.2) Solubility of dPC and oleic acid in the three incubation mixtures

Samples containing dPC with ^{3}H-dPC and ^{14}C-oleic acid in either solution 1, solution 2 or solution 3 were incubated at 37°C for 30 minutes in order to mimic the incubation conditions to be used. The aqueous samples were then aspirated and the recovery calculated by comparison with ^{3}H-dPC and ^{14}C-oleic acid dispensed directly into scintillation vials .

From tables 3 & 4 it can be seen that both Tween-80 and Triton X-100 markedly increase the solubility of both dPC and oleic acid into aqueous solution. Tween-80 increased the solubility of dPC from 67% to 98% and oleic acid from 18% to 67% whilst solution 3 improved solubility of dPC from 67% to 86% and oleic acid from 18% to 91%. However marked variability in the solubility using solution 2 was evident when compared to solution 3.

Table 3) Recovery of ^{3}H-dPC from Sarstedt tubes after incubation at 37°C for 30 minutes (mean +/- s.e.m.)

Buffer	Control DPM	Aqueous DPM	% Recovery
Tris (solution 1)	16672.4 +/- 209.6	11191.6 +/- 204.7	67 % n=4
Tris & 0.1% Tween -80 (solution 2)	10720.9 +/- 144.2	10519.2 +/- 103.3	98 % n=4
HEPES & Triton X-100 (solution 3)	26273.0 +/- 36	22814.0 +/- 40.2	86 % n=4

Table 4) Recovery of ^{14}C-oleic acid from Sarstedt tubes after incubation at 37°C for 30 minutes (mean +/- s.e.m.)

Buffer	Control DPM	Aqueous DPM	% Recovery
Tris (solution 1)	18308.3 +/- 1204.2	3372.6 +/- 1714.3	18 % n=4
Tris & 0.1% Tween -80 (solution 2)	13914.8 +/- 574.0	12073.5 +/- 1211.7	86 % n=6
HEPES & Triton X-100 (solution 3)	10639.0 +/- 102.3	9422.9 +/- 98.0	91 % n=4

6.3) The effect of equilibration of silica gel with solvent 1 over time, on the retention of dPC and oleic acid by mini-columns

It was found that heat activation of the silica gel for over 30 minutes followed by equilibration in solvent 1 for periods of over a week, resulted in improved recovery of dPC and oleic acid from mini-columns. 150ul of Tris-HCl (pH 7.5) containing 50 nmols (ie 333uM) dPC, ^{3}H-dPC (150,000 DPM) and ^{14}C-oleic acid (83,000 DPM) was added to minicolumns which had been prepared using silica which had or had not been heat activated and which had been equilibrated over a period of days in solvent 1. Silica retained more activity when it was not heat

activated (see figure 35) and retention was sustained to 6 days. Retention of dPC and oleic acid was reduced when the silica gel was heated and this reduction increased further over time. (see table 5). All columns used were thus equilibrated for 10 days or more in solvent 1 prior to packing into the mini-columns.

Table 5) The effect of heat activation and equilibration of 100-200 mesh silica gel in solvent 1 on the retention of dPC and oleic acid

	% Recovery of activity from mini-columns			
Equilibration (Days)	Heated		Not Heated	
	dPC	oleate	dPC	oleate
1	2.8	5.6	3.5	5.2
2	34.1	37.2	20.0	26.1
6	20.6	61.8	3.0	6.2
10	59.5	77.8	-	-

All colmuns were thus equilibrated for over 10 days.

6.4) Separation and extraction of dPC and oleic acid using mini-columns

The advantage of this technique developed by Consentino & Legrand, (1981) is that it extracts and separates dPC and oleic acid within one step. To characterise this, four 150ul samples of solutions 2 and 3 containing dPC (333uM), ^{3}H-dPC and ^{14}C-oleic acid were passed through four mini-columns which had been equilibrated for 7 days in solvent 1 . From figure 36 it can be seen that extraction of dPC and oleic acid from solution 2 into solvents 1 and 2 respectively was 106 +/- 6.9% and 63.1 +/- 1.6%. Extraction from solution 3 was 97.0 +/- 10.34 % (dPC) and 121 +/- 9.12 % (oleic acid).

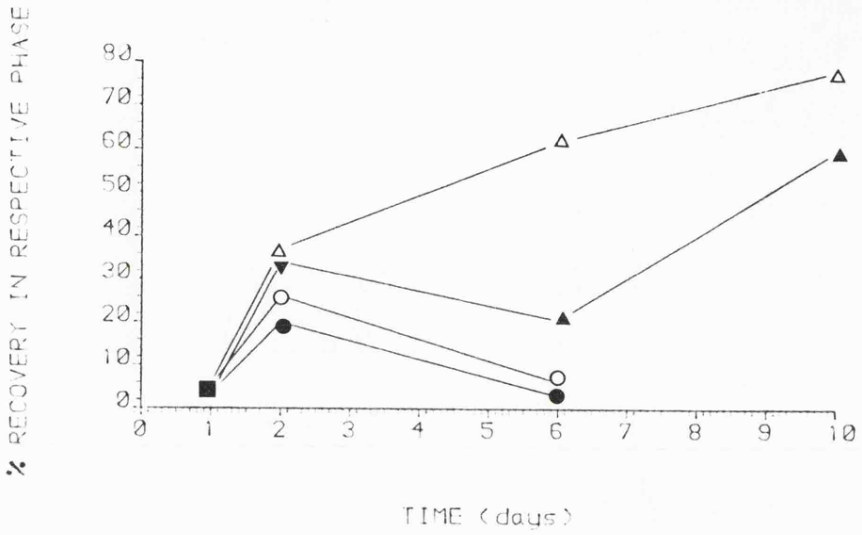
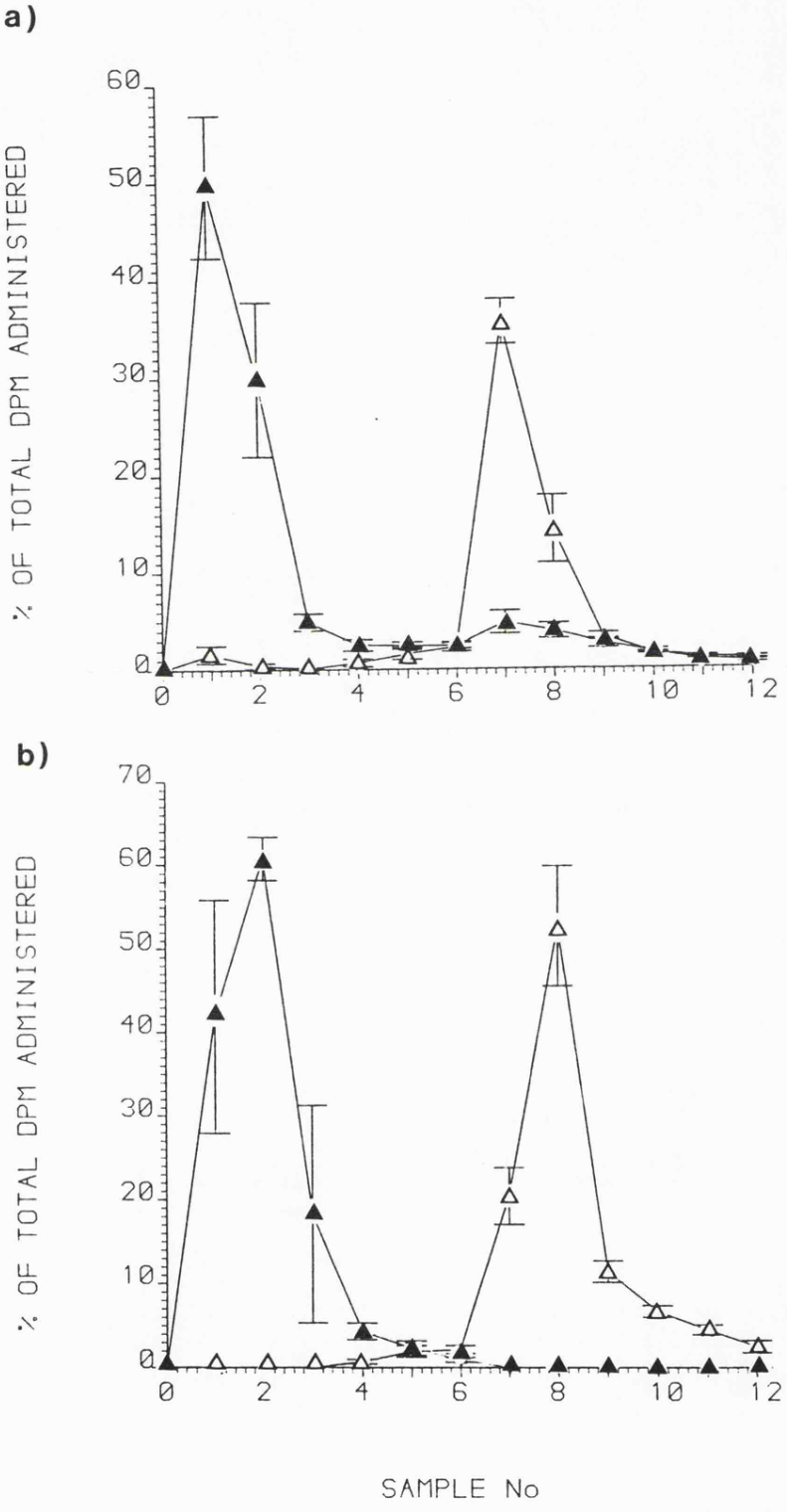


Figure 35) The effect of time of equilibration of 100-200 mesh silica gel in hexane:dioxane:glacial acetic acid (70:30:1, solvent 1) (and heating prior to equilibration) on the retention of ^{14}C-oleic acid (open symbols) and 1-oleoyl,2(^{3}H-oleoyl)-phosphatidylcholine (closed symbols) in mini-columns. Silica gel was heated to 100^{o}C (triangles) or not heated (circles) prior to equilibration in solvent 1. The samples were applied in Tris buffer (50mM pH7.5). Oleate was eluted in 1ml samples (1-6) by solvent 1 and dPC in chloroform:methanol:water (65:35:1 , solvent 2). Retention was reduced at 10 days with the heated columns but full recovery of oleic acid and dPC was not attained.

Figure 36) The separation and extraction of ^{14}C-oleic acid (▲) and 1-oleoyl,2-(^{3}H-oleoyl)-phosphatidylcholine (^{3}H-dPC, △) suspended in tris buffer (100 mM, pH 7.5) with 0.1% Tween 80 (solution 2, figure a) and in HEPES buffer (50 mM) with 0.2% Triton-X100 (solution 3, figure b) using mini-columns containing heated 100-200 mesh silica gel equilibrated for 10 days in hexane:dioxane:glacial acetic acid (70:30:1, solvent 1). Oleate was eluted in 1ml samples (No's 1-6) by solvent 1 and dPC in 1ml samples (No's 7-12) in chloroform:methanol:water (65:35:4). From solution 2 106.0 +/- 6.9 % of oleic acid and 63.1 +/- 10.3% dPC was recovered. From solution 3 121.0 +/- 9.1% of oleic acid and 97.0 +/- 10.3% of dPC was recovered. Points and vertical lines indicate mean +/- s.e.m.(n=4).


a)
% OF TOTAL DPM ADMINISTERED
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50
40
30
20
10
0
0
2
4
6
8
10
12
b)
% OF TOTAL DPM ADMINISTERED
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
SAMPLE No

Separation of dPC and oleic acid was complete with both solutions. Oleic acid was eluted in solvent 1 and dPC in solvent 2. 95.8 +/- 1.7% of dPC eluted was present in solvent 2 and 86.3 +/- 2.0% of oleic acid eluted was present in solvent 1 when solution 2 was used. Of the total dPC eluted from the solution 3 , 95.0 +/- 1.3% dPC was in solvent 2 whilst 98.3 +/- 0.1% oleic acid was in solvent 1.

Solution 3 was then used throughout all subsequent procedures since the solubility of dPC and oleic acid was more consistent and no dPC was retained by the column when compared with solution 2.

6.5) Extraction of dPC and oleic acid prior to separation byTLC

Aqueous samples cannot be applied to silica plates for separation of constituents. Therefore several methods were assessed for solvent extraction of dPC and oleic acid from solution 3 by vortexing with a range of organic solvents (Tables 6 & 7). Results were compared to paired aqueous samples added directly to Aqua Luma. n-Heptane at pH 11.0 was the most efficient at extracting the dPC and oleic acid, however the extract solidified on contact with the HEPES buffer and was therefore not used.

Table 6) Extraction of ^{3}H-dPC from aqueous samples (solution 3) into organic solvents at pH 3.0 and 11.0

Solvent	pH 3.0	pH 11.0
n-Heptane	86.1 %	93.0 %
Chloroform	1.5 %	2.4 %
diChloromethane	1.9 %	3.3 %
diethylether	29.7 %	63.7 %
Butan-1-ol	0.4 %	0.3 %

Table 7) Extraction of ^{14}C-oleic acid from ageous samples (solution 3) into 1ml of organic solvents at pH 3.0 and 11.0

Solvent	pH 3.0	pH 11.0
n-Heptane	44.9 %	98.1 %
Chloroform	0.7 %	8.4 %
diChloromethane	0.7 %	54.9 %
diethylether	1.9 %	38.8 %
Butan-1-ol	0.5 %	0.5 %

6.6) Extraction of dPC and oleic acid using the method of Bligh and Dyer (1959)

This technique relies on the phase separation of chloroform from the methanolic layer on saturation with water. Extraction at 98.0 % (n=4) of dPC and 93.0 % (n=4) of oleic acid was good, however it was noted that on the second washing with chloroform that the lipids often came out of solution. Addition of 50ul of methanol ensured solubility after extraction. This method of extraction was used for all experiments involving subsequent TLC separation.

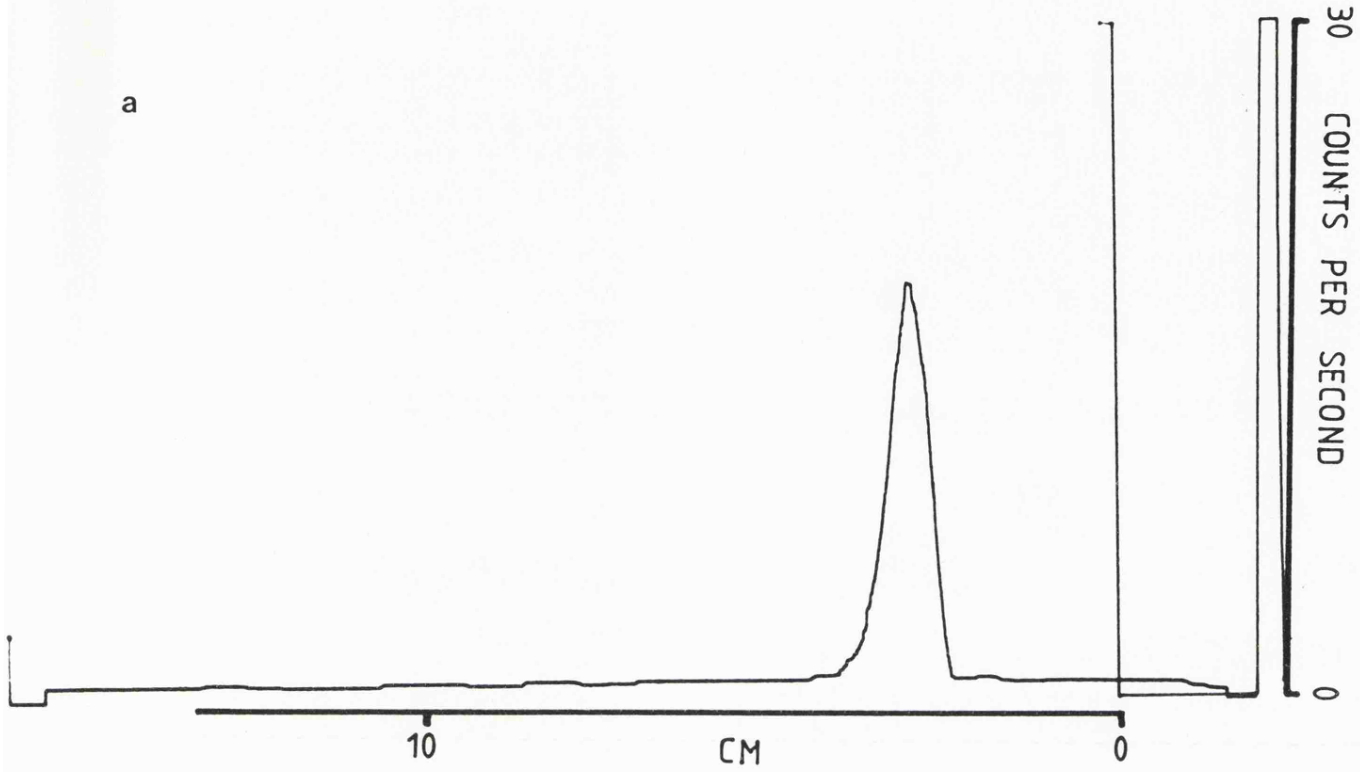
6.7) Separation of dPC and oleic acid using TLC

In order to ascertain the profile of fatty acid and glycerophospholipid separation using TLC samples containing ^{3}H-dPC and ^{14}C-oleic acid in toluene were applied to TLC plates and run in solvent 3. Plates were then scanned (see figure 37). Oleic acid travelled with the solvent front and dPC was retained on the plate (rf = 0.33). L~-lysophosphatidylcholine applied in a similar manner was also retained on the plate (rf = 0.15). On scraping and counting by liquid scintillation,

Figure 37) Thin-layer chromatograms from a Berthold TLC scanner (30 sec time constant , 50mV sensitivity , scanning speed 300mm/hr). TLC plates were developed in solvent 3 (Chloroform : methanol : glacial acetic acid : water , 50 : 15 : 4 : 2 , v/v/v/v).

a) 200 nMols dPC containing ^{3}H-dPC was applied to the origin (O) and developed for 10cm. rf=0.33.

b) ^{14}C-oleic acid was applied to the origin (O) and developed for 10cm. The oleic acid ran with the solvent front.


a
30
COUNTS PER SECOND
0
10
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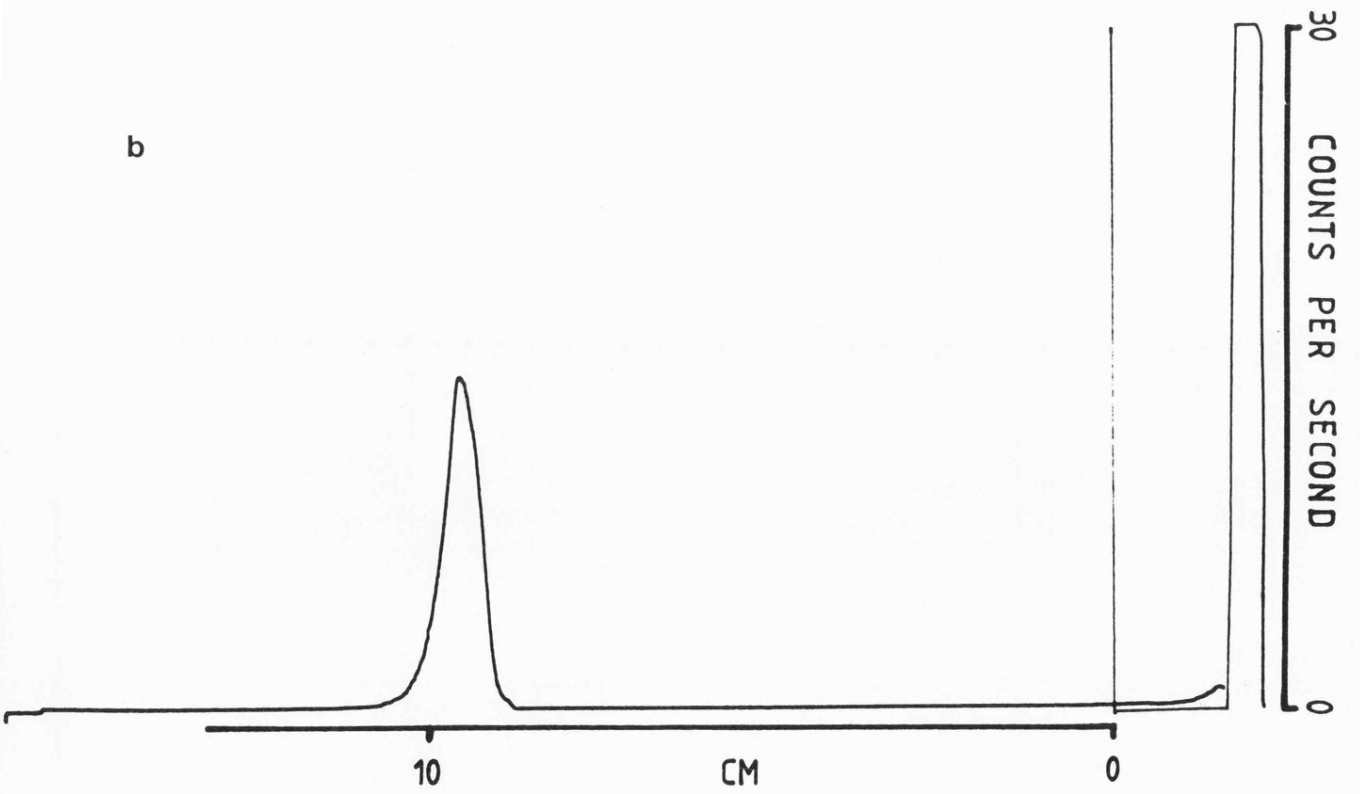

b
30
COUNTS PER SECOND
0
10
CM
0

99.7% of ^{14}C-oleic acid was present in the final 2cm of the lane and 99.0% of ^{3}H-dPC was present in the first 8.0cm.

6.8) Elution of ^{3}H-dPC and ^{14}C-oleic acid from TLC plates

^{3}H-dPC and ^{14}C-oleic acid were applied to separate lanes of TLC plates in concentrations which would be used experimentaly and developed in solvent 3. The silica from each lane was taken up in methanol or directly into scintillation fluid and the activity compared with samples applied directly to the scintillation vials. 45.6% ^{3}H-dPC and 97.5% ^{14}C-oleic acid were recovered without methanol and 95.0% ^{3}H-dPC and 94.0% ^{14}C-oleic acid with methanol. Further experiments showed that 94.4% of ^{3}H-dPC (n=4) was recovered with prior treatment with methanol and 97.3% of the ^{14}C-oleic acid (n=4) was recovered when the ^{3}H-dPC was extracted using scintillant alone. For this reason dPC was extracted from the silica in methanol and oleic acid in scintillant alone.

6.9) Action of authentic phospholipase-A_2 on ^{3}H-dPC

The action of authentic porcine pancreatic PLA_2 was determined to assess the performance of the two methods of separation and the position of the label on the ^{3}H-dPC. Figure 38 shows the action of authentic PLA_2 (5 U/ml) on the substrate (solution 3) and subsequently separated using mini-columns. 5 U/ml PLA_2 in 150ul of solution 3 released 82.5% of the activity into solvent 1 (figure 38). Figure 39 shows samples under the same incubation conditions except separated using TLC after extraction by the method of Bligh & Dyer (1959). 97.3% of all activity was released and ran with the solvent front indicating ^{3}H-dPC cleavage.

The importance of sonication of the substrate mixture is also

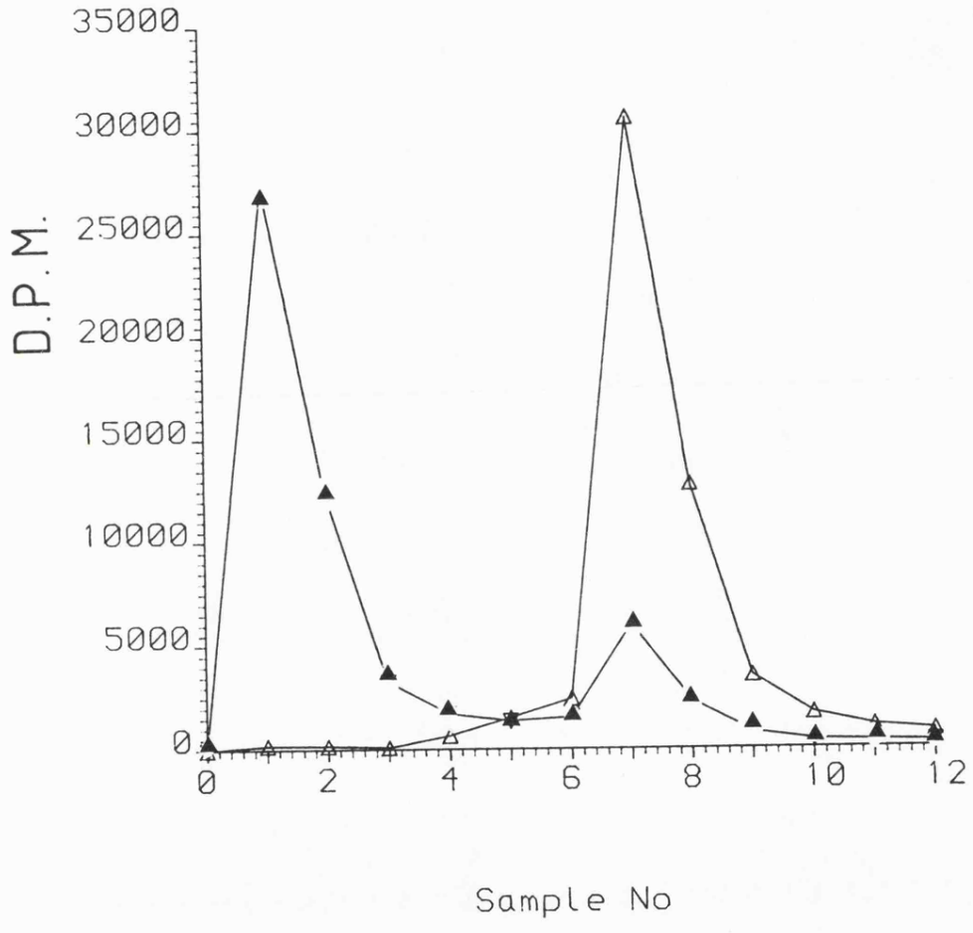


Figure 38) The effect of authentic phospholipase-A_2 [PLA_2 ▲]on 1-oleoyl,2(^{3}H-oleoyl)-phosphatidylcholine (dPC △; Mini-column separation). dPC and released oleic acid were separated on mini-columns packed with 100-200 mesh silica. 1ml samples in tubes 1-6 were eluted in hexane:dioxane:glacial acetic acid (70:30:1 , solvent 1) and tubes 7-12 in chloroform:methanol:water (65:35:4 , solvent 2). PLA_2 cleaved 85% of the ^{3}H-oleic acid from the ^{3}H-dPC.

illustrated in figure 39. Sonication of solution 3 ensured complete liberation by authentic PLA_2 whilst the absence of sonication resulted in only 41.2% conversion under the same conditions.

6.10) Time course of ^{3}H-oleic acid release and the comparison of separation of dPC and oleic acid by mini-column and TLC after incubation with rat uterine homogenate

Rat uterine homogenate was incubated with 333uM ^{3}H-dPC and paired 150ul samples were taken over time from the same vessel and separated by TLC and mini-column. The resulting time courses (figure 40) show completely different profiles. The accumulation of ^{3}H-oleic acid indicated by TLC is sharp and peaks after one hour at 6.88nmols oleate released per mg of protein whilst the mini-column indicated only a slow accumulation peaking after 30 minutes at 2.91 nmols/mg protein.

The separation of released oleic acid by TLC is more complete than by mini-column as the extent of conversion of dPC by authentic PLA_2 indicated by mini-column is incomplete, unlike TLC. This could lead to inaccuracies in the calculation of PLA_2 activity and thus TLC was taken as the method of choice for all further experiments.

6.11) The effect of phospholipase-A_2 inhibitors on the PLA_2 activity in pregnant rat uterine homogenates

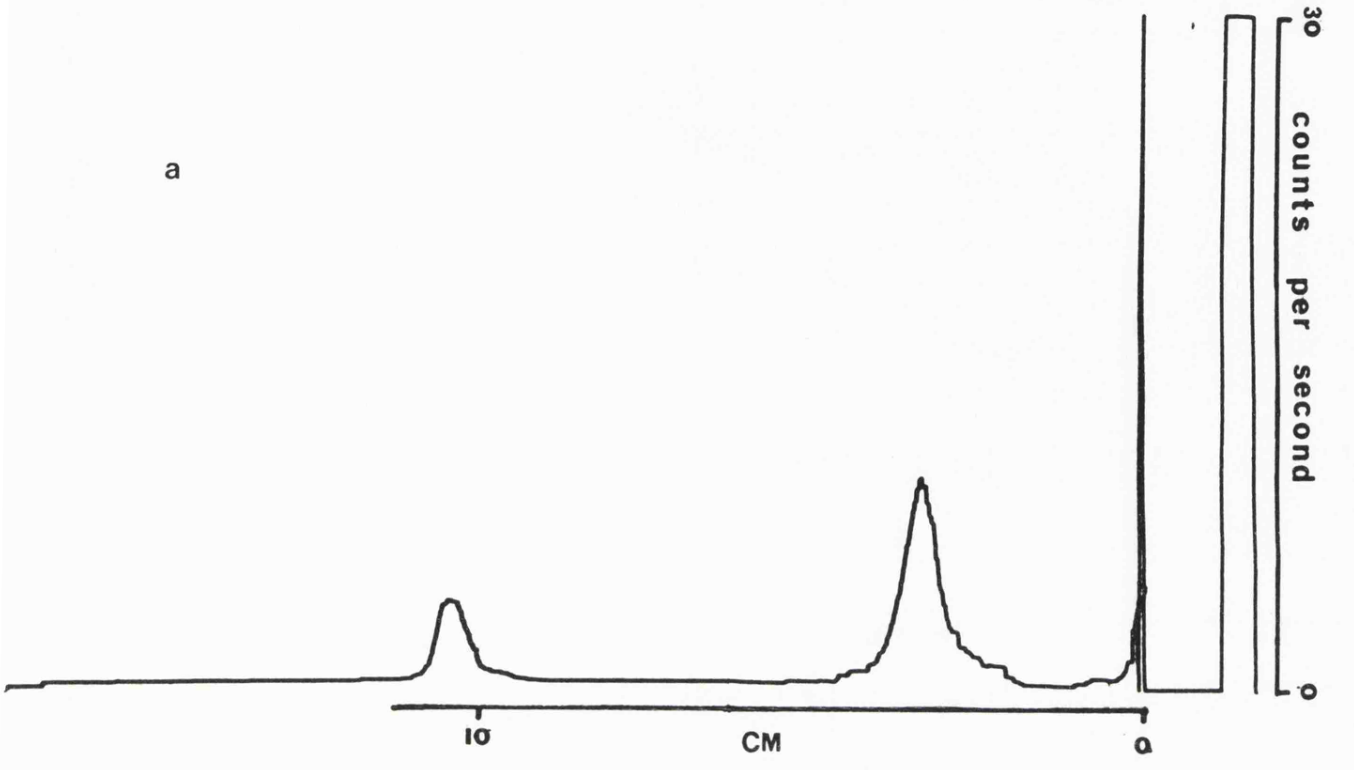
Incubation of pregnant rat uterine homogenate (day 19) with solution 3 in the presence of mepacrine (6.77uM) and tetracaine (1mM) resulted in the complete inhibition of ^{3}H-oleic acid release (see figures 41 & 42) when compared with uterine homogenates incubated without mepacrine or tetracaine.

Figure 39) Thin-layer chromatogram scanned with a Berthold scanner (30 second time constant, 50mV sensitivity, scanning speed 300mm/hour), illustrating the effect of authentic PLA_2 on 1-oleoyl,2-(^{3}H-oleoyl)-phosphatidylcholine prepared with or without sonication. TLC plates were were developed in solvent 3 (Chloroform : methanol : glacial acetic acid : water , 50 : 15 : 4 : 2 , v/v/v/v)

a) 333uM dPC with ^{3}H-dPC incubated in solution 3 (NOT SONICATED) at 37^{o}C with authentic PLA_2 (5U/ml) for 30 minutes.

b) 333uM dPC with ^{3}H-dPC incubated in solution 3 (SONICATED) at 37^{o}C with authentic PLA_2 (5U/ml) for 30 minutes.

Sonication resulted in the release of 97.3% of ^{3}H-oleic acid from the substrate as compared to 41.2% without sonication.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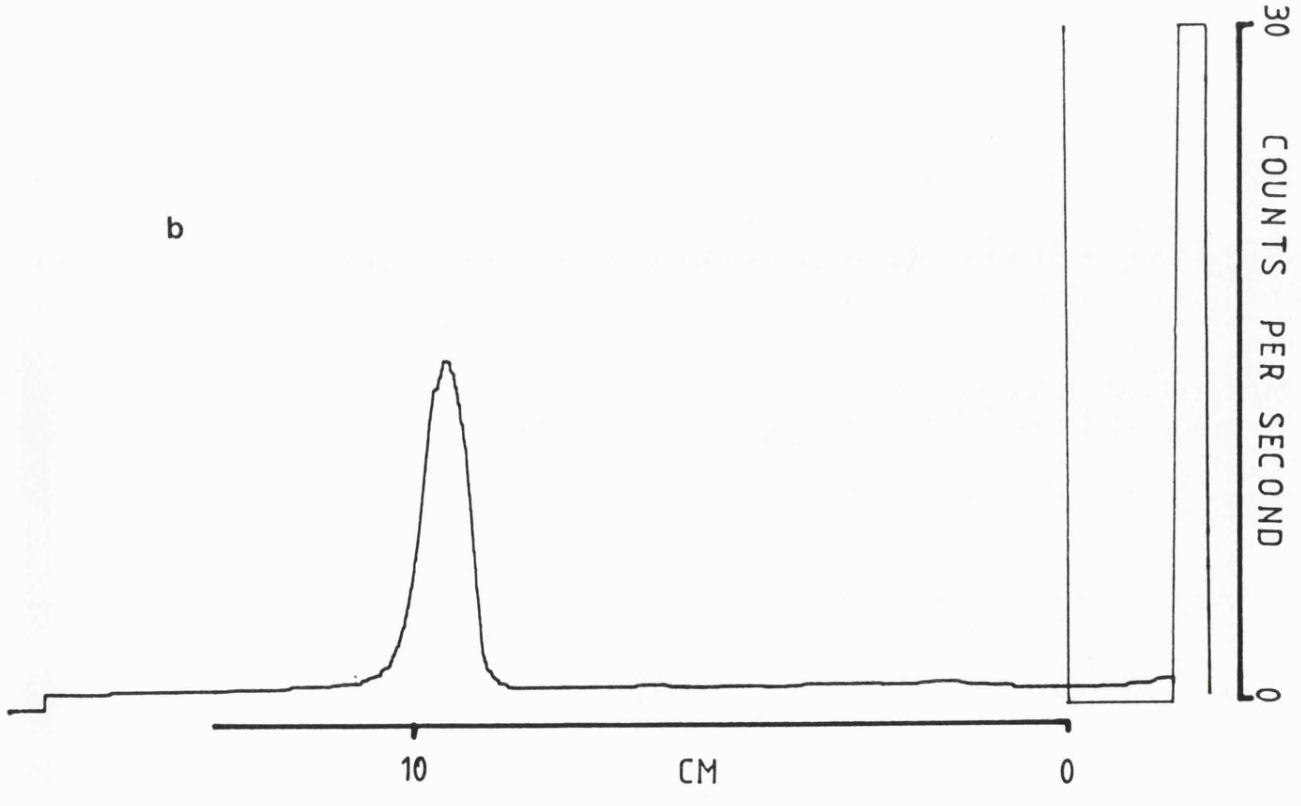
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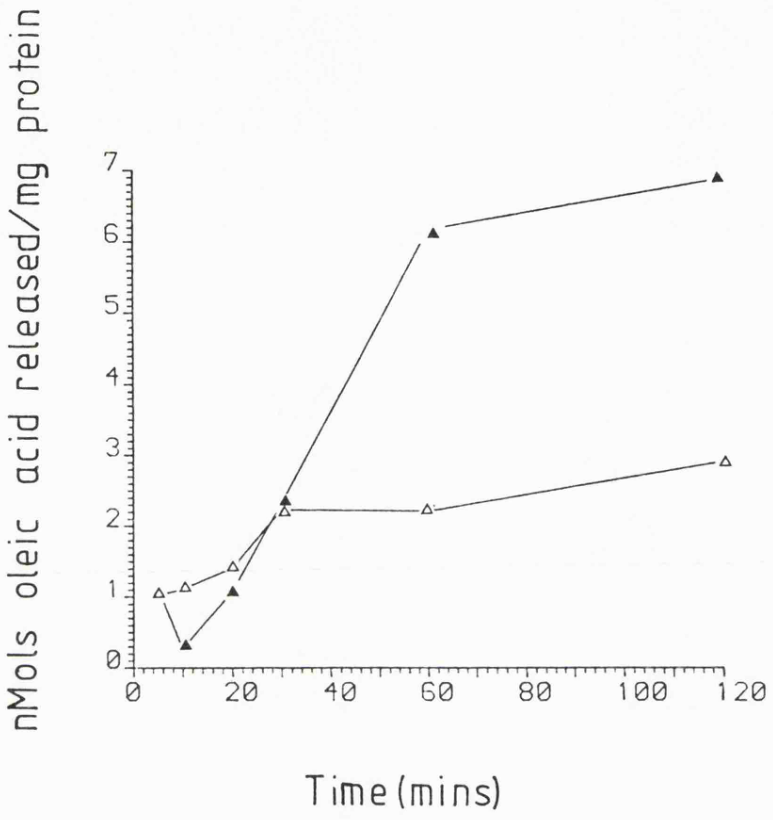

Figure 40) Paired comparison of indicated conversion of 1-oleoyl,2-(^{3}H-oleoyl)-phosphatidylcholine (dPC) by the 700g supernatant of a rat uterine homogenate (day 19 pregnant) using two methods of product and substrate extraction and separation. Released ^{3}H-oleic acid was extracted by the method of Bligh & Dyer (1964) and separated from dPC by thin-layer chromatography (TLC, ▲) and compared to extraction and separation by mini-column (△).

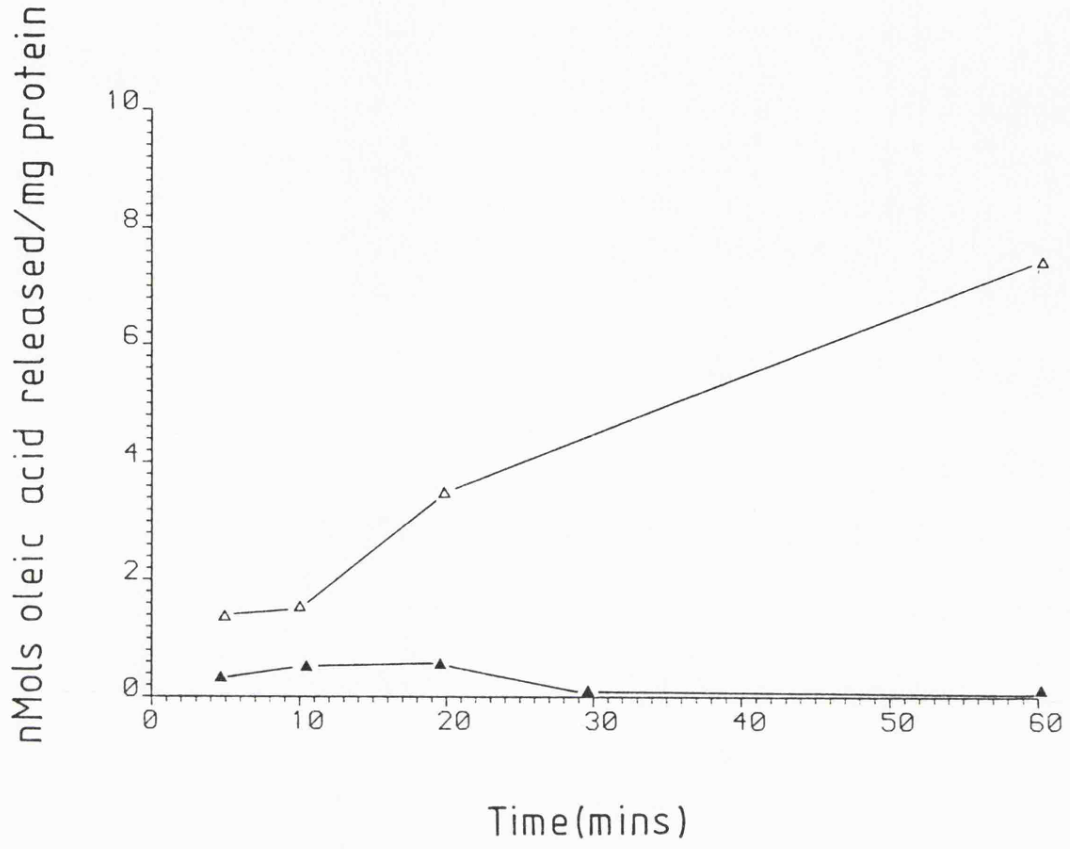

Figure 41) The effect of mepacrine (6.77mM, ▲) on the release of oleic acid by the 700g supernatant of a day 18 pregnant rat uterine homogenate (△). Mepacrine abolished the the release of ^{3}H-oleic acid from 1-oleoyl,2-(^{3}H-oleoyl)-phospatidylcholine.

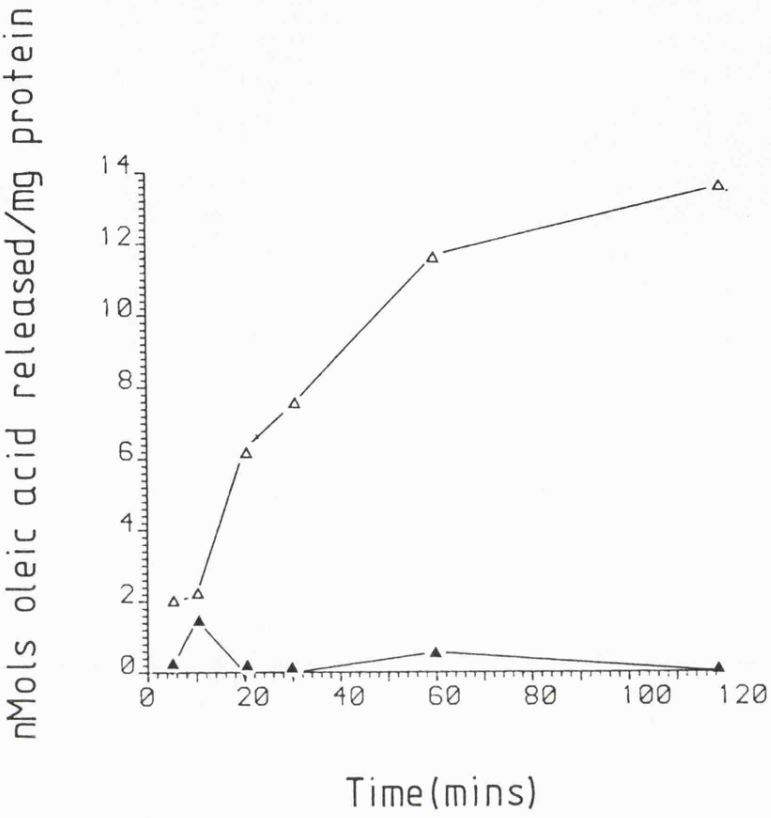

Figure 42) The effect of tetracaine (▲;1mM) on the release of oleic acid by the 700g supernatant of a day 19 pregnant rat uterine homogenate (△). Tetracaine abolished the release of ^{3}H-oleic acid from 1-oleoyl,2-(^{3}H-oleoyl)-phosphatidylcholine.

6.12) Protein dependance of the release of oleic acid by the rat uterine homogenate

Three substrate mixtures (solution 3) were prepared with different concentrations of rat uterine homogenate. The concentrations were calculated as 1.488 (boiled control and high protein concentration sample) and 0.744 mg/ml. The incubation mixtures were incubated at 37°C for 30 minutes and samples taken at 5, 10, 20 and 30 minutes. The release of oleic acid increased with protein concentration (see figure 43).

The enzyme rate was calculated for 0.744mg/ml solution as 2.468 and for the 1.44mg/ml solution as 2.410 nmols/mg protein/hour (figure 44). Higher concentrations of protein were not used as the buffering capacity of the substrate mixture may not have been sufficient at this level. The ratio of 1 part homogenate : 5 parts substrate (i.e. 17% homogenate/substrate) was used for all experiments.

6.13) pH optimum for ^{3}H-oleic acid release from ^{3}H-dPC by pregnant rat uterine homogenate

Substrate mixtures of solution 3 were prepared over a range of pH 6.75 to 8.5 and the rate of release of ^{3}H-oleic acid after incubation with the uterine fraction calculated over 20 minutes for two 20 day pregnant rats. A peak of 29.88 at pH 8.0 as compared to 13.62 nmols/mg/hour at pH 7.5 (see figure 45). All subsequent experiments were carried out at pH 8.0.

6.14) The effect of calcium on ^{3}H-oleic acid release from ^{3}H-dPC by pregnant rat uterine homogenate

Substrate mixtures (solution 3) containing 0 - 10mM $CaCl_2$ were

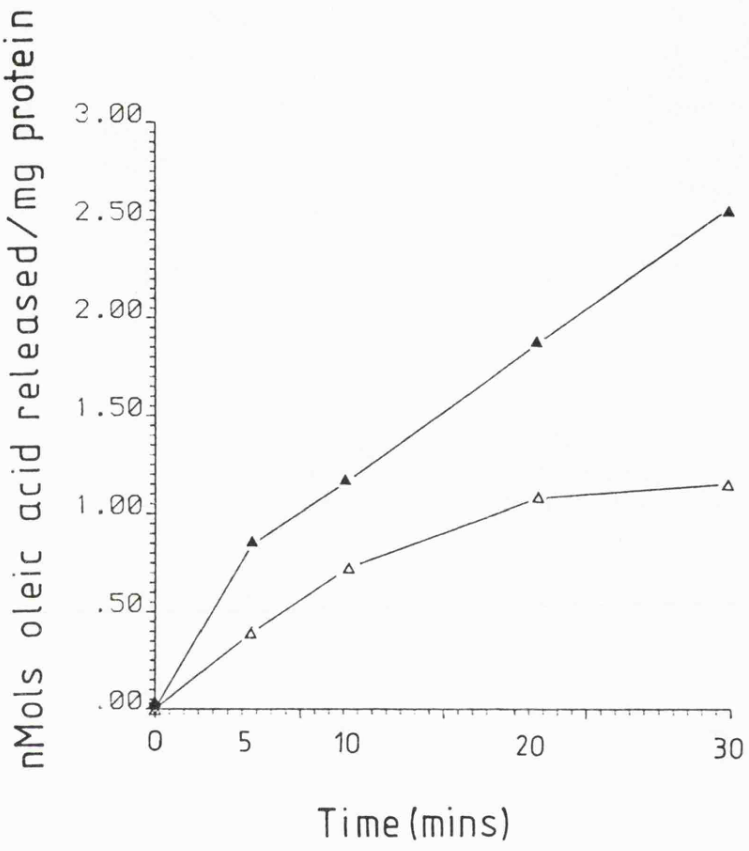

Figure 43) The effect of protein concentration on the release of ^{3}H-oleic acid from 1-oleoyl,2-(^{3}H-oleoyl)-phosphatidylcholine by the 700g supernatant of a day 19 pregnant rat uterine homogenate. (▲ : 1.48 mg/ml; △ : 0.744 mg/ml). The reaction is protein dependant .

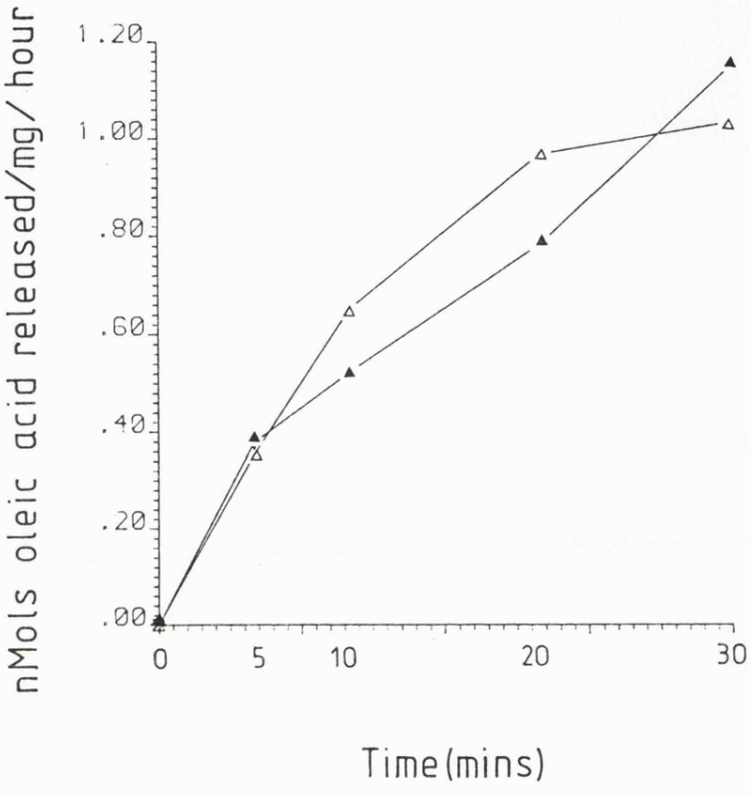

Figure 44) The effect of protein concentration on the rate of release of ^{3}H-oleic acid from 1-oleoyl,2-(^{3}H-oleoyl)-phosphatidylcholine per mg of protein by the 700g supernatant of a day 19 pregnant rat uterine homogenate over time of incubation. The rate of conversion per mg protein over time is not affected by protein concentration. (▲: 1.48 mg/ml; △: 0.744 mg/ml).

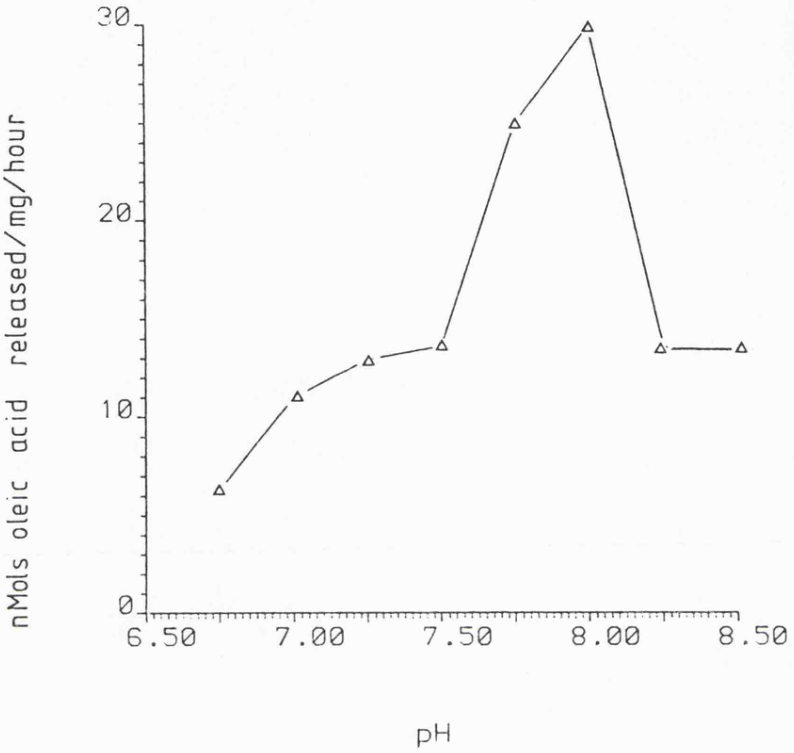

Figure 45) The effect of pH on the rate of release of ^{3}H-oleic acid from 1-oleoyl,2-(^{3}H-oleoyl)-phosphatidylcholine (dPC) by the 700g supernatant of a day 20 pregnant rat uterine homogenate. Release of ^{3}H-oleic acid was maximal at pH 8.0.

prepared and 150ul samples taken at 5, 10 and 20 minutes. Figure 46 indicates that although there was no graded stimulation of activity related to calcium concentration, the release of oleate by rat uterine homogenate had an absolute requirement for calcium.

6.15) The effect of Substrate concentration on the release of ^{3}H-oleic acid from ^{3}H-dPC by pregnant rat uterine homogenate

Substrate mixtures (solution 3) were prepared with dPC ranging from 41.6 to 1332uM, and the rate of conversion by day 20 pregnant rat uterine homogenate was calculated from progress curves constructed from samples taken at 5, 10 and 20 minutes. The conversion was substrate dependant tending to reach a maximum above 1332uM (figure 47 ; Vmax= 16.8 nmols/mg/hour, Km= 0.7845) and 1/2 Vmax at 480uM dPC.

For all future experiments duplicate samples were used at a substrate concentration of 1/2 Vmax , consisting of 17% homogenate : 83% substrate mixture (solution 3) at pH 8.0 . Duplicate samples were taken at 20 minutes of incubation since the correlation coefficient for those values calculated from data using 5, 10 and 20 minute samples compared to 20 minutes samples was 0.99 (n=23).

6.16) The effect of perfusion of the uterine bed on the calculated enzyme activity

It was noted that uteri taken from late pregnant rats contained significant amounts of blood trapped within the myometrium and deciduum. This would affect the protein assay to give a higher protein concentration and thus result in a lower calculated enzyme activity. One horn of a 19 day pregnant rat was perfused with Krebs' solution as

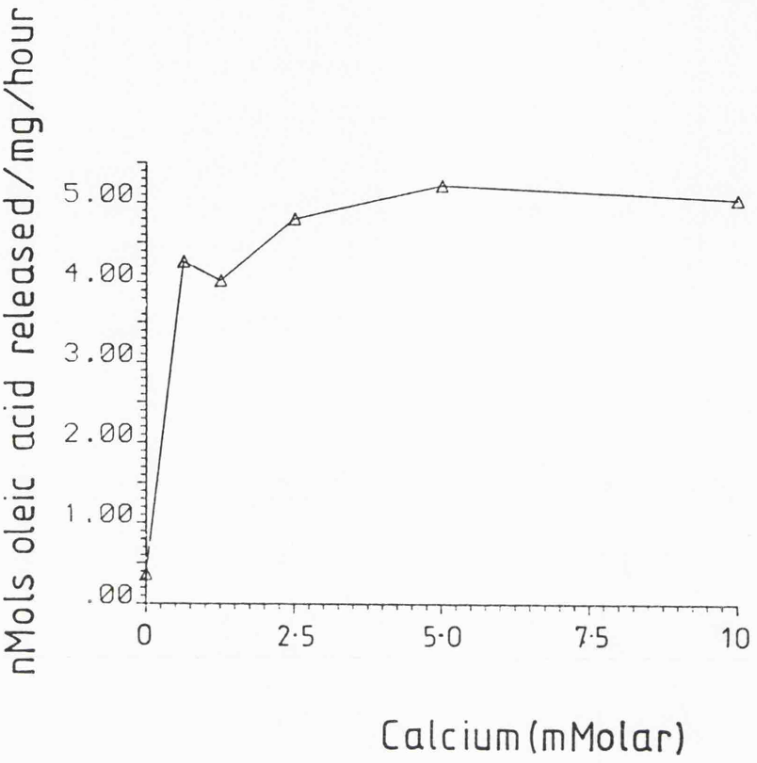

Figure 46) The effect of calcium concentration on the rate of release of ^{3}H-oleic acid from 1-oleoyl,2-(^{3}H-oleoyl)-phosphatidylcholine (dPC) by the 700g supernatant of a day 19 pregnant rat uterine homogenate. dPC was only liberated in the presence of calcium.

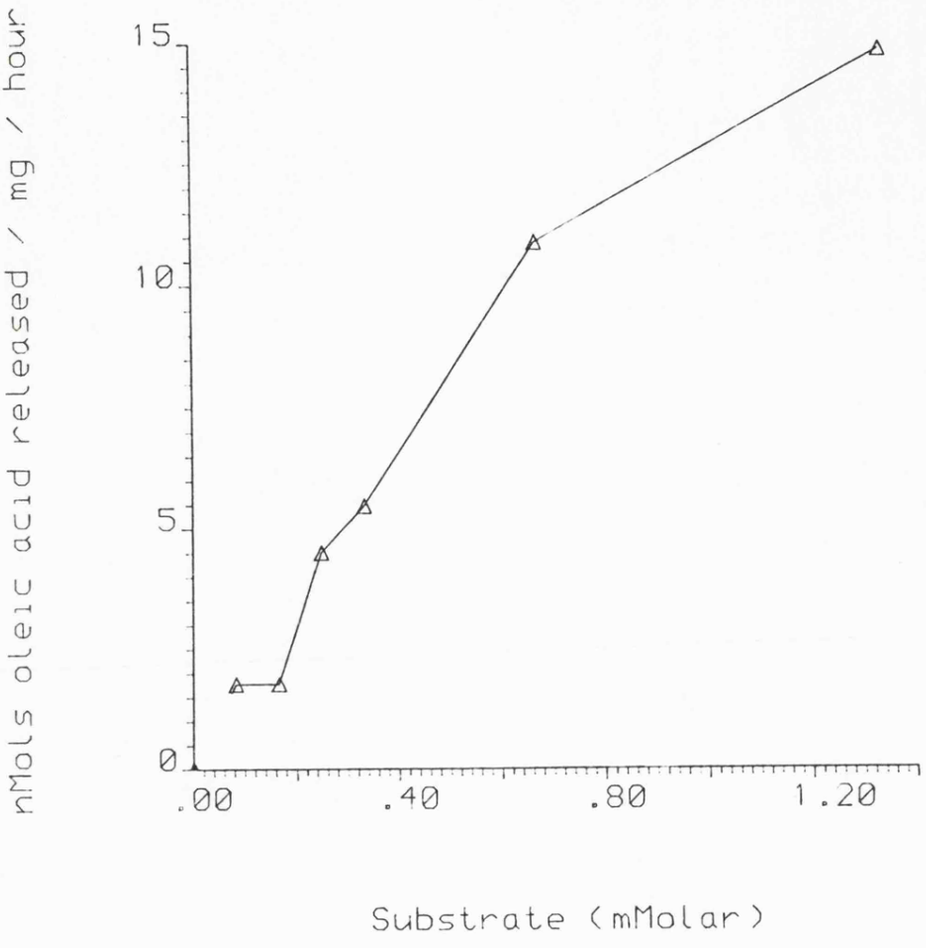

Figure 47) The effect of substrate concentration on the rate of release of ^{3}H oleic acid from 1-oleoyl,2-(^{3}H-oleoyl)-phosphatidylcholine (dPC) by the 700g supernatant of a day 20 pregnant rat uterine homogenate. The rate of conversion was dependant on the substrate concentration. Vmax = 16.8 nMols/mg/hr, Km = 0.7845.

described in section 2.2 and the PLA_2 activity assayed.

The calculated PLA_2 activity was increased from 28.5 to 40.3 nmols/mg/hour on perfusion. The protein concentration assayed was reduced from 1.97 to 0.78 mg/ml. Therefore all future experiments used perfused uterine tissue. The dry weight/wet weight ratio of the uterine tissue was also reduced by perfusion from 7.3% to 3.2%.

6.17) The contribution of myometrial and decidual PLA_2 to the whole uterine PLA_2

One uterine horn from a day 19 pregnant rat was perfused with Krebs' solution and divided into two portions. One portion was incubated as normal and the decidua scraped off the other using a microscope slide. The myometrium and the deciduum fromthe same sample were incubated in the volume which the whole tissue would require. The activity was assessed at 20 minutes incubation time.

The whole uterus had the capacity to liberate oleic acid at the rate of 40.3 nmols/mg/hour. The myometrium and decidua released 18.3 and 24.4 nmols ^{3}H-oleic acid/mg/hour respectively (a total of 42.7 nmols/mg/hour). The myometrium therefore contributed 42.8% to total activity when assessed in this way.

7.0) DISCUSSION

Optimal conditions for the release of anti-aggregatory material from the human myometrium (Bamford et al., 1980), placenta (Myatt & Elder,1977) and rat myometrium (ElTahir & Williams, 1980) have already been determined. The conditions for myometrial release were corroborated, however the conditions for the release from the placenta differed substantially from those reported by Myatt & Elder (1977). Anti-aggregatory activity was only released after preincubation at 37°C for 10 minutes followed by chopping and further incubation at 22°C for 15 minutes, and no activity was released without preincubation or chopping. This could indicate that incubation at 37°C results in activation of or release of this material into the intracellular medium, and is then released into the incubation medium on chopping.

Rat myometrium separated from deciduum by scraping and incubated after chopping produced substantial release of PGI_2-like anti-aggregatory material reaching a maximum at 15 minutes incubation at 22°C in TBS. This agrees with the results obtained by ElTahir & Williams (1980) using Krebs' solution. However the release was observed to be markedly altered by tissue handling , the method of removal of decidual tissue affecting maximal synthesis and the rate of release being altered by chopping. The fact that chopping increased the rate of output of PGI_2-like material but not maximal release may indicate the presence of a negative feedback mechanism as myometrial PGI_2 stimulates cAMP formation (Vesin, Do Khac & Harbon, 1979; Omini, et al., 1979) and PGI_2 synthesis may be altered by intracellular cAMP (see later). Removal of decidual tissue from the rat myometrial tissue excludes interference due to the anti-aggregatory activity of PGD_2 and PGE_2 which are predominantly synthesised by the deciduum (Harney, Sneddon & Williams,

1974; Williams & Downing, 1977; Abel & Kelly, 1979; Alwachi, Bland & Poyser, 1980).

Rat and human myometrial anti-aggregatory activity released into the incubation medium behaved in a similar manner to authentic PGI_2 in that they were stable at alkaline pH, but acid labile; properties known to be peculiar to PGI_2 amongst the PGs. Both PGD_2 and PGE_2, anti-aggregatory PGs known to be released by the myometrium (Vane & Williams, 1973; Katori, Harada, Yamashita, Ishibashi & Miyazaki, 1978), retained activity after acidification but this was lost on storage at alkaline pH. This is presumably due to the conversion of PGE_2 to PGB and PGD_2 to a more polar substance (Pace-Asciak, 1976b). PGI_2-free human myometrial incubate did not affect the anti-aggregatory activity of PGI_2. Furthermore, the anti-aggregatory potencies of PGD_2 and PGE_2 were very low since rabbit platelets are relatively insensitive to these PGs when compared to PGI_2, unlike human platelets (Whittle, Monacada & Vane, 1978).

Placental anti-aggregatory activity on the other hand was not abolished on alkalinisation but attenuated. Mild acidification to pH 7.5 from pH 8.0 with incubation at 22°C did not alter placental activity, unlike the myometrial activities and authentic PGI_2 which were reduced. Pretreatment of tissues with indomethacin inhibits the release of activity from rat (ElTahir & Williams, 1980) and human (Bamford et al.,1980) myometrial tissues whilst leaving placental activity unaffected (Myatt & Elder, 1977). Whilst these results show that the material released from the myometrial tissues is probably PGI_2, the placental substance(s) does not possess the same properties and exhibits a different time course of anti-aggregatory action against ADP-induced

aggregation to both the myometrial activities and PGI_2.

Chow, Craft, Dandona and Hutton (1980) reported the release of an ADPase into TBS from chopped placental tissue when incubated at 37°C which reduced the aggregatory activity of ADP . Therefore ADP was incubated at room temperature with myometrial and placental extracts, very little ADP degrading activity was noted in the human myometrial incubate unlike the human placental incubate which contained significant activity. Although human myometrial incubates have been reported to contain an ADPase (Hutton, Chow, Craft & Dandona, 1980) when incubated at 37°C, the ADP degrading capacity of the human myometrium was low at 22°C . It was thought that the contribution of ADPase to human myometrial PGI_2 anti-aggregatory activity under these conditions was insignificant for the purposes of this study. However the ADPase activity present in the placental incubate would have completely overshadowed any anti-aggregatory activity exhibited by PGI_2 and this was not pursued further. The alkaline lability of the placental activity may have indicated an effect by PGD_2 or PGE_2, which are synthesised in quantity by the placenta (Demens & Gabbe, 1976; Mitchell, Kraemer & Strickland, 1982). However, since indomethacin did not even reduce this activity at doses that inhibited the release of myometrial anti-aggregatory activity, this is unlikely.

The fact that the anti-aggregatory activity released under these conditions by the placenta is not PGI_2 does not preclude the possibility that PGI_2 is synthesised by the placenta. 6-oxo-$PGF_{1\alpha}$ specific RIA suggests that there is endogenous release of PGI_2 from placental tissue (Mitchell, Bibby, Hicks & Turnbull, 1978).

The assay coefficients of variation for the assay of the PGI_2 content of human myometrial incubation media were low, as were those for

rat myometrium (ElTahir & Williams, 1980). However species differences in the sensitivity of platelets to PGI_2 has led to the widespread use of human platelets. Since these are 3-5 times more sensitive to PGI_2 than rat or rabbit platelets respectively (Whittle et al., 1978) platelets pretreated with papaverine were used for samples containing low levels of PGI_2.

Papaverine was anti-aggregatory at high doses and potentiated the anti-aggregatory activity of authentic PGI_2 in a dose related fashion. Maximal potentiation of PGI_2 activity by papaverine was found at the subthreshold dose of 100uM. This also potentiated the anti-aggregatory effects of myometrial and PGI_2 anti-aggregatory activities to the same extent and did not reveal any other acid stable anti-aggregatory activity (eg. PGD_2 and PGE_2). This potentiation was used as an accurate and sensitive extension to the assay of anti-aggregatory activity in human myometrial incubates since the coefficients of variation were low and similar to those of the unmodified assay. Papaverine probably potentiated anti-aggregatory effects by providing a sub-maximal inhibition of platelet phosphodiesterase, since 50uM papaverine is reported to inhibit human platelet phosphodiesterase by 50% (Mills & Smith, 1971). PGI_2 elevates platelet cAMP levels (Tateson, Moncada and Vane, 1977; Gorman, Bunting & Miller, 1977) via receptor activation (Mills & Macfarlane, 1977). Thus the degradation of the pulse of cAMP generated by PGI_2 would not be so efficient and the anti-aggregatory activity thus potentiated. The inhibition of platelet aggregation by PGE_1 can be potentiated by several phosphodiesterase inhibitors including theophylline, papaverine and dipyridamole (Mills & Smith, 1971). In turn the inhibition of platelet aggregation by PGI_2 is

potentiated by PGE_1, theophylline and dipyridamole (Whittle et al., 1978) using doses which in themselves are not anti-aggregatory.

Further evidence that the anti-aggregatory activity released by human myometrium is an AA metabolite was found on incubation of human myometrial tissue with AA or PLA_2. Both substances stimulated PGI_2 formation to a similar degree. This supports the thesis that PG synthesis is limited by AA availability (Kunze & Vogt, 1971), and the report that AA and PLA_2 stimulate PGI_2 synthesis by the rat pregnant myometrium (ElTahir & Williams, 1980). Thus the rate of PGI_2 synthesis is not dependent on low cyclo-oxygenase or PGI_2 synthetase activities or the lack of precursor but at an earlier step, probably PLA_2. Indomethacin also inhibited human myometrial PGI_2 synthesis presumably by the inhibition of cyclo-oxygenase (Ahern & Downing, 1970; Vane 1971). High concentrations were used as reported in previous work (Bamford et al., 1980) and would indicate the presence of a cyclo-oxygenase enzyme with low affinity for inhibition by non-steroidal anti-inflammatory drugs (NSAID). Flower and Vane (1974) have reported different susceptibilities of the cyclo-oxygenase enzymes in different tissues to inhibitors. The possibility of a low affinity for NSAID by uterine cyclo-oxygenase in late pregnancy has already been suggested (Terragno & Terragno, 1981). The potency of indomethacin in inhibiting dog uterine PG formation is reduced ten-fold from that required to inhibit non-pregnant uterine formation (Terragno et al., 1974; Terragno, Terragno & McGiff, 1976). Also the dose of indomethacin required to inhibit totally non-pregnant rat myometrial PGI_2 synthesis is <5 ug/ml whilst that required to inhibit pregnant rat myometrial PGI_2 release by 50% is 20 ug/ml (ElTahir & Williams, 1980) under the same incubation conditions.

The proliferation of an enzyme characterised by low NSAID affinity

may account for these differences. Cyclo-oxygenase activity is known to increase during pregnancy in rat uterine tissue (Williams & Vane, 1973) and myometrium (Williams & El Tahir, 1980a) presumably in preparation for the activation of PLA_2 at term.

As well as being synthesised by the myometrium, PGI_2 was found to alter human myometrial contractility. PGI_2 irreversibly inhibited the spontaneous contractility and reduced the basal tone of the lower segment myometrium in vitro. Omini et al. (1978, 1979), Sanger & Bennett (1980) and Wilhelmsson, Wikland & Wiquist (1981) have reported a reduction in spontaneous motility and basal tone in both pregnant and non-pregnant human myometria. Even though complete inhibition was not seen, Omini and coworkers (1979) elicited an inhibitory effect at 2-20 ng/ml in the superfused pregnant myometrium whilst 200ng/ml (Omini et al., 1978) was required in superfused non-pregnant myometrium; 100ng/ml (Sanger & Bennet, 1980) and 1000 ng/ml (Wilhelmsson et al., 1981) abolished contractility in non-pregnant tissue bathed in Krebs' solution. The present study shows that the inhibitory potency of PGI_2 differs not because of the preparation of the myometrium during pregnancy but because of the in vitro conditions. Superfusion with Tyrode's solu tion and a constant infusion of PGI_2 to the superfusion fluid (Omini et al., 1978) as opposed to bathing in Krebs' solution probably results in a greater availability of PGI_2 to the tissue prior to hydrolysis to 6-oxo-$PGF_{1\alpha}$. However in this study the effect of PGI_2 was profound and irreversible, whereas contractility is resumed after the infusion of PGI_2 is stopped (Omini et al. 1978, 1979). Dusting, Angus and leDuc (1982) have noted a similar irreversible effect with epicardially superfused PGI_2 on circumflex coronary artery blood flow

which remains unexplained. The mechanism of action of PGI_2 in causing uterine relaxation is probably via an increase in myometrial cAMP levels (Omini et al. 1979) which is a common feature with other uterine relaxants.

PGI_2 also elicited a slight increase in the contractility of the pregnant myometrium in 2/4 preparations, prior to relaxation. An initial report of an oxytocic action of PGI_2 on human myometrium (Wilhelmsson, Lindblom & Wiquist, 1979), was attributed to a 'batch' difference in the PGI_2. However Sanger & Bennet (1980) reported that 30% of tissues initially contracted to PGI_2. Intravenous infusion of PGI_2 at low doses (<10 ng/kg/min) in non-pregnant women has no effect on uterine contractility (Makainen & Ylikorkala, 1982) whereas intra-uterine instillation of PGI_2 (0.3 - 0.6 ug) caused a gradual stimulation of activity. These studies probably illustrate an oxytocic action of PGI_2 at high concentrations, and a tocolytic action at lower concentrations. Bolus or high doses of PGI_2 (1000 ng/ml) to superfused human myometrium elicited contractions and lower doses reduced contractility (Omini et al., 1978). Similar findings have also been reported in the human umbilical artery (Pomerantz, Sintetos & Ramwell, 1978), rat (Levy, 1980) and rabbit thoracic aorta via the synthesis of an unstable pro-aggregatory vasoconstrictor similar to TxA_2 (Borda, Sterin-Borda, Gimeno, Lazzarri & Gimeno, 1983).

The observation that human myometrium has the ability to synthesise increasing amounts of of PGI_2 during the last two weeks of pregnancy is in agreement with studies in the rat (Williams & ElTahir, 1980a) but the aamounts are 10 times lower in human tissue . The exact mechanism for this increase is unknown, but the rise in rat and human myometrial PGI_2 synthesis is coincident with a peak in plasma oestradiol levels

(Anderson, 1977; Wilson, Stanisc, Kahn-Dawood & Dawood, 1982; Turnbull , Patten, Flint, Keirse, Jeremy & Anderson, 1974). Plasma oestradiol increases during pregnancy in women peaking at weeks 30-40 (Dawood & Ratnam, 1974; Turnbull et al., 1974). Free and bound plasma progesterone increases also throughout pregnancy (Yannone, Mueller & Osborne, 1969) and has been reported to fall before labour (Csapo, Poharka & Kaihola, 1974).

There is no data available at present on the effects of steroids on pregnant human myometrial PGI_2 synthesis. Oestradiol has been found however to stimulate 6-oxo-$PGF_{1\alpha}$ release by cultured term-placental cells, whilst progesterone inhibits this action (Myatt, Jogee & Elder, 1983). The action of oestradiol was thought to be either by the activation of PLA_2 and/or inhibition of PGDH at higher doses. Oestrogens also increase the synthesis of PGs in rat uterine tissue (Ryan, Clark, Van Orden & Brody, 1974) and passive immunisation against oestradiol significantly reduces the concentration of PGE_2 and $PGF_{2\alpha}$ in rat uterine venous blood in labour (Csapo, Currie, Erdos & Resch, 1978), whilst NSAID inhibit oestradiol-stimulated PG formation by rat uterus (Jordan & Castracane, 1976). This stimulation of PG synthesis has been tentatively attributed to an increase in PLA_2 activity. In order to investigate the effects of steroids and other factors an assay for rat uterine PLA_2 activity was characterised.

Enzymic activity which liberated ^{3}H-oleate from ^{3}H-dPC was isolated in the pregnant rat uterus. The methods of separation of the hydrolysis products was found to be critical. Although mini-column separation as described by Consentino and Legrand, (1981) resulted in a good separation of the released fatty acid from the glycerophospholipid,

conversion detected by this method was less than that found using TLC as the separative procedure. Lyso-phosphatidylcholine can affect the mobility of fatty acids in similar systems and could explain this disparity. TLC was therefore used as the method of choice.

The activity within the pregnant rat uterus exhibited absolute calcium dependance, an alkaline pH optimum, and was susceptible to inhibition by mepacrine and tetracaine, both inhibitors of phospholipase activity (Marcus & Ball, 1969; Kunze et al., 1974). These properties infer a phospholipase-dependent release of oleate and several mechanisms should be considered: 1) release of oleate from the sn1 position by PLA_1 and migration of the lyso-PC with the oleate at the solvent front, 2) release of oleate by PLA_1 from the sn1 position with subsequent breakdown of the liberated lyso-PC by a lyso-phospholipase, 3) the action of PLC with subsequent liberation of ^{3}H-oleate by the action of 1,2-diacylglycerol-lipase on phosphatidic acid (PA), 4) the action of PLC with the liberation of ^{3}H-oleate by a PA dependent PLA_2 and 5) the release by PLA_2 from the sn2 position.

To take the first two points, PLA_1, lipases and phospholipases do not require calcium (McMurray & Magee, 1972; Gatts & Barebholz, 1973) and secondly bovine seminal vesicle lipases are inhibited by high (10mM) calcium concentrations (Kunze et al., 1974). Also the mobility of the lyso-PC liberated by PLA_1 has a similar mobility to PC in the TLC system used. Thirdly, PLC derived from platelet and human foetal membranes is specific for PI (Lapetina & Cuatrecasas, 1979; DiRenzo, Johnstone, Okazaki, Okita, MacDonald & Bleasedale, 1981) and not PC which was used in this study. It is therefore probable that the enzyme is PLA_2, and not PA-specific PLA_2 since the latter has a pH optimum of 7.0 (Billah, Lapetina, Cuatrecasas, 1981). More conclusive evidence would be to use

dual-labelled dPC in order to illustrate hydrolysis at either or both the snl and sn2 positons and test for inhibition or activation by EDTA since EDTA inhibits PLA_2 but stimulates lipase activity (Kunze et al., 1974). Examples of the use of this assay system were then illustrated.

The release of ^{3}H-oleate by day 19 pregnant rat uterus under optimal conditions was high at 40 nMols/mg/hour. Uteri taken from hypophysectomised rats convert 2.2 nMols/mg/hour (Dey et al., 1982) whilst at day 1 of pregnancy this activity is only 4.1, peaking at implantation on day 4 at 15.8 and returning to 6.61 nMols/mg/hour on day 6 (Cox, Cheng & Dey, 1982). This peak in activity corresponds with a reported peak in serum oestradiol (Watson & Alam, 1976) and uterine oestradiol receptor concentration (Glasser & McCormack, 1981). Oestradiol pretreatment of hypophysectomised rats results in a considerable increase in uterine PLA_2 activity (Dey et al., 1982) to levels similar to day 19 of pregnancy. The oestradiol surge by increasing PLA_2 activity could thus explain the increased release of PGI_2 from the rat myometrium noted at day 21 over day 19.

The fact that oestradiol and progesterone are capable of modifying PG synthesis may indicate a receptor mediated activation/inactivation of AA release. The uterine stimulants oxytocin and BK both stimulate rat pregnant myometrial PGI_2 synthesis (Williams & ElTahir, 1980 b) which is in itself a stimulant of rat uterine contractility (Omini et al. 1977; ElTahir et al., 1979). Oxytocin, angiotensin II, ergometrine and 5-HT all increased the synthesis of PGI_2 by rat pregnant myometrium in a dose related manner, and the effect of 5-HT was antagonised by methysergide, a 5-HT receptor antagonist (Awouters, Leysen, De Clerk & Van Nueten, 1982). The PLA_2 inhibitor mepacrine reduces the stimulated output by

these drugs whilst leaving the stimulant activity of AA unaffected (Williams et al., 1983) suggesting they are acting via increasing PLA_2 activity . PLA_2 is activated by calmodulin (Walenga, Opas & Feinstein, 1981), and as such could be controlled by intracellular processes which modulate intracellular calcium such as changes in cAMP concentrations (reviewed by Bar, 1974). This is supported by the findings that bradykinin (Arold, Leibman, Roemer, Paegelow & Reissman, 1976), oxytocin (Bhalla, Sunborn & Koreman, 1972) and angiotensin II (D'Aurac, Angles & Mayer, 1972) reduce rat uterine cAMP levels, whilst 5-HT (Tereschenko, Kurski & Fedorov, 1972), angiotensin II (Blackmore, Dehaye & Exton, 1979), oxytocin (Carsten, 1974) and ergometrine (Giovanni & Albero, 1966) reduce Ca^{++} uptake and binding by myometrial fractions, which causes an increase in intracellular calcium.

On the other hand, salbutamol, a specific β_2 adrenoreceptor agonist, reduced PGI_2 synthesis by the 20 day rat pregnant myometrium. Although propranolol tended to reduce PGI_2 synthesis, salbutamol did not reduce synthesis further to those levels seen without propranolol, indicating receptor antagonism. However salbutamol did not inhibit PGI_2 synthesis by myometria from 19 day pregnant rats. Salbutamol reduced the synthesis of PGI_2 in the day 21 myometria to that seen on day 19 of pregnancy. This could indicate either an action on stimulated PGI_2 synthesis in the oestrogen dominated myometrium or be a result of an increase in β receptor dependent cAMP production which is known to occur in late pregnancy (Rinard & Chew, 1978). Since relaxin and histamine, both uterine relaxants (Porter, Downing, & Bradshaw, 1979; Kameswaran et al., 1962), reduced PGI_2 synthesis by rat 19 day pregnant myometrium (El Tahir et al., 1983) it would seem that the latter is true.

The intracellular events following beta receptor activ .ation are well known (Schultz & Jacobs, 1982). β_2 adrenoreceptor stimulation results in the relaxation of the non-pregnant rat uterus which is antagonised by propranolol and to a much lesser extent practolol (Wasserman & Levy, 1972). Uterine cAMP levels increase on administration of isoprenaline which is blocked by propranolol; potentiated by theophylline and mimicked by dibutyryl-cAMP (Robinson, Butcher & Sutherland, 1968). Adrenaline and dibutyryl-cAMP both inhibit oxytocin induced contractions (Triner, Overweg & Natas, 1970) whilst dibutyryl-cAMP abolishes calcium-induced contractions (Mitznegg, Heim & Meythaler, 1970). Such stimulation of β_2 receptors of the rat uterus is accompanied by a parallel increase in the intracellular cAMP binding (Harbon, Do Khac & Vesin, 1976) and protein kinase activation (Harbon, Vesin, Khac & Leiber, 1978).

Vascular tone is controlled in a similar manner (Bohr, Greenberg & Bonaccarsi, 1978). Hydralazine, a potent hypotensive agent (Wilkinson, Backman & Hecht, 1952) was also found to inhibit pregnant rat myometrial PGI_2 synthesis. The fact that hydralazine has been reported to relax rat myometrium at concentrations over 10^{-4}M (Worcel, Saiag & Chevillard, 1980) and is therapeutically beneficial as a tocolytic in pre-eclamptic patients (Bowman & Rand, 1980) as well as a hypotensive agent supports the thesis that uterine relaxants reduce PGI_2 synthesis by the rat pregnant myometrium.

Thus oxytocic drugs would reduce intracellular cAMP via receptor-mediated inhibition of adenylate cyclase, and increase intracellular calcium resulting in activation of PLA_2, PG release and myometrial contraction. Indeed, inhibition of cyclo-oxygenase reduces the oxytocic

efficacy of oxytocin (Vane & Williams, 1973), angiotensin II (Baudouin-Legros et al., 1974) and 5-HT (Sorrentino et al., 1972). Drugs which reduce uterine contractility would act in the reverse manner by increasing intracellular cAMP.

Further evidence for the involvement of cAMP in regulating myometrial PGI_2 synthesis comes from the observation that forskolin inhibited pregnant rat myometrial PGI_2 synthesis. Forskolin is a diterpene which selectively activates a sub-unit of adenylate cyclase (Seaman & Daly, 1979). Although its action on rat myometrium has not been reported, there are reports that forskolin has smooth muscle relaxant activity (Seaman & Daly, 1983).

It is generally accepted that smooth muscle cell activation is accompanied by a rise in cytoplasmic calcium from 10^{-7} to 10^{-5} molar. PLA_2 is generally activated by millimolar concentrations of calcium (see van den Bosch, 1980) whilst PLC responds to micromolar concentrations (Mauko, Chap & Douste-Blazy, 1979; Allan & Mitchell, 1974). PLA_2 however could be activated by calmodulin (Wong & Cheung, 1979; Walenga, Opas & Feinstein, 1981) whilst PLC is not (Walenga et al., 1981). Calmodulin present in uterine muscle (Grand & Perry, 1978) could thus increase the activity of lower concentrations of calcium in stimulating uterine PLA_2.

A recent, though controversial, hypothesis for receptor controlled release of AA from glycerophospholipids involves the concept of the increase in PI turnover and PLC activation (Lapetina, 1982; Chap, Laffont, Lensta, Mauco, Perret, Platavid, Simon, Thomas & Douste-Blazy, 1983) and has been characterised mainly using platelets.

The activation of PLC results in the formation of 1,2,diacylglycerol from PI. The action of 1,2,diacylglycerol kinase forms PA and lyso-PA from 1,2,diacylglycerol before and after the action of lipases. PA and

lysoPA are both calcium ionophores and release calcium from cardiac sarcoplasmic reticulum (Limas, 1980) and platelet membrane fractions (Gerrard, Butler, Petersen & White, 1976) whilst they also cause platelet aggregation (Benton, Gerrard, Michiel & Kindom, 1982; Tokunua, Fukuzawa, Isob & Tsukatani, 1981) and may mediate contraction in smooth muscle (Salmon & Honeyman, 1980). Since they possess these properties, PA and lysoPA may stimulate the liberation of AA via activating PLA_2 (Apitz-Castro, Cruz, Mas & Jain, 1981; Lapetina, Billah & Cuatrecasas, 1981) and mimic calmodulin (Niggli, Adunyah & Carafoli, 1981). Differences in tissues may exist since activation of PI specific PLA_2 by bradykinin in pig aortic endothelial cells precedes that of PLC (Hong & Deykin, 1982). However care must be taken in interpreting these results since at has recently been shown that PA degradation products may mediate PA-induced release of Ca^{++} from PC liposomes (Holmes & Yoss, 1983).

It is interesting to note that human amnion, chorion laevae and decidua vera contain PI specific PLC (DiRenzo, et al., 1981), 1,2,diacylglycerol lipase (Okazaki, Sagawa, Okita, Bleasedale, MacDonald & Johnstone, 1981) and 1,2-diacylglycerol kinase (Okita, Sagawa, Casey & Snyder, 1983). PI and PE are both reduced in labour in women, PE more so than PI (Okita, MacDonald & Johnston, 1980) therefore the release of AA into the amniotic fluid at term (MacDonald, et al., 1974) arises from either the activation of PLA_2 alone or with PLC. Since 1,2,diacylglycerol is released into the amniotic fluid during parturition it would be of interest to observe PA levels and correlate this with AA release.

The contradict ory actions of the vasodilators dipyridamole

(increase) and hydralazine (decrease) on rat myometrial PGI_2 synthesis are puzzling since both drugs have many aspects in common. Both stimulate aortic PGI_2 synthesis (Greenwald, Wong, Alexander & Bianchine, 1980); exhibit anti-phosphodiesterase activity (Mills & Smith, 1971; Inatomi, Takoyonaki & Takagi, 1975); possess anti-platelet activity (Bologna, 1972; Pfister & Imhof, 1979); inhibit platelet TxA_2 synthesis (Greenwald, Wong, Rao, Bianchine & Panganamale, 1979) and stimulate PGI_2 production by vascular smooth muscle cells (van de Velde, Bult, Weisenberger & Herman, 1982; Dyer, Huttner, Tan & Mulrow, 1981). Hydralazine stimulates the synthesis of vasodilatory PGs in tissues other than the uterus, such as rat aorta, brain and renal medulla (Taube, Hauser, Duneman & Foerster, 1978). Because of these actions these workers ascribe a role for PGI_2 in the vasodilatory and anti-hypertensive actions of these drugs.

This difference must be found in their mechanism of action and whether these drugs are affecting the same pool of enzymes involved in PGI_2 synthesis. A direct comparison of the effect of dipyridamole and hydralazine on the metabolism of exogenous AA by rat lung and aorta shows that while dipyridamole stimulates 6-oxo-$PGF_{1\alpha}$ release by both tissues, hydralazine inhibits lung synthesis and tends to stimulate aortic 6-oxo-$PGF_{1\alpha}$ release (Srivastava & Alwachi, 1983). This illustrates that there are tissue differences in the action of these drugs on AA metabolism.

Hydralazine has been reported to cause an accumulation of cAMP in rabbit aortic tissue (Andersson, 1973) and reduces calcium uptake by vascular tissue (Mclean, du Souich, Barron & Briggs, 1978). Noradrenaline -induced release of intracellular calcium is also reduced in the presence of hydralazine (Mclean, Barron, du Souich, Haegele,

McNay, Carrier & Briggs, 1978) and would explain the reduction of PGI_2 synthesis by pregnant rat myometrium by hydralazine.

The action of dipyridamole on PG synthesis has been pursued since it was found to stimulate PGI_2 synthesis in the human forearm (Masotti et al., 1979). At a concentration of 100uM, dipyridamole stimulates cyclo-oxygenase and the conversion of PGH_2 to PGE_2 by aortic microsomes (Mentz, Ponicke, Block, Blass, Geissler & Forster, 1980). This is supposedly unrelated to its anti-oxidant activity (Morisaki, Stitts, Bartels-Tomei, Milo, Panganamala & Cornwell, 1982) or its anti-phosphodiesterase activity since these enzymes are unaffected by cyclic nucleotides.

There have been no reports of a direct effect of dipyridamole on PLA_2 activity however. Indirect evidence comes from observations that dipyridamole stimulates PGI_2 synthesis from endogenous AA by umbilical vessels (Menta, Menta & Hat, 1980) human forearm (Masotti et al., 1979), and rat aorta (van de Veld et al., 1982). Although it has been shown that drugs such as thrombin and bradykinin stimulate 3T3 fibroblast cyclo-oxygenase in a manner which can contribute significantly to the stimulation of PG synthesis (Bonser, Cgandrebose & Cuatrecasas, 1980), this effect has no action without the induction of PLA_2 to release excess quantities of AA.

The issue is further complicated by the finding that the stimulation of PGI_2 synthesis of the pregnant rat myometrium was not sustained by dipyridamole at 400uM. This reduction in stimulation could indicate an inhibition of cyclo-oxygenase at these concentrations as has been noted by Bult, Herman & van de Velde (1982). Another factor which could contribute to this reduction of PGI_2 synthesis at high concentrations of

dipyridamole is the action of the vehicle, ethanol. Ethanol reduced basal synthesis at all concentrations used. Ethanol administration in premature labour in women is tocolytic (Fuchs, Fuchs, Poblete & Risk, 1961) and has been found to act on the myometrial membrane to reduce the effect of oxytocin (Mantell & Liggins, 1970).

It must be noted that in all the experiments using uterine stimulants and relaxants, relatively high concentrations were used compared to those required to inhibit or stimulate uterine contractility in vitro. This may be a result of differences in the physiological state of the preparation and the fact that the PG synthesising capacity of the tissues was studied as opposed to PG content. The alteration of PGI_2 production is not as a result of membrane distortion as a result of drug treatment since $PGF_{2\alpha}$, a potent uterine stimulant, does not affect myometrial PGI_2 output (Williams & El Tahir, 1978b).

The whole uterus consists of two tissues, the myometrium and deciduum. The effects of any drugs would depend on the interrelationship of the two and relative effects of the drugs on the two. The release of PGs by the two fractions differs. The distribution of PLA_2 within the pregnant rat uterus was not found to be homogeneous. The decidua, which consists of less than 30% of the whole tissue, contained 57% of the total uterine PLA_2 activity. A similar distribution of PLA_2 activity has been reported for both human and ovine uterine tissues (Grieves & Liggins, 1976). The decidua contains higher levels of AA than the myometrium (Filshie and Anstey, 1978) and has the ability to release substantial quantities of PGF, PGD and PGE (Williams & Downing, 1977). Abel and Kelly (1979) provided evidence that the endometrium of the non-pregnant human uterus donates AA to the myometrium for metabolism to PGI_2 at the cost of PGF and PGE synthesis. However it must be noted that

stimulation of PGI_2 release from the human myometrium by PLA_2 and AA resulted in synthesis equivalent to that of the 40 week pregnant myometrium, and not more. This suggests that donation of AA would only affect PGI_2 synthesis up to the thirty ninth week of pregnancy. Evidence for the donation of AA by the decidua to the myometrium has not been found in the pregnant rat (El Tahir & Williams, 1981), in fact the opposite was reported to occur, i.e. myometrial PGI_2 synthesis was inhibited by a platelet-derived lipoxygenase product released from the decidua. After removal of trapped blood elements there was no stimulation of myometrial PGI_2 synthesis by the decidua. Campos, Liggins and Seamark (1982) have confirmed that there is no AA donation by the deciduum in the rat. They separately superfused endometrial and myometrial layers in the whole non-pregnant rat uterus and assayed the PG content within the superfusate. PGF and 6-oxo-$PGF_{1\alpha}$ production from both layers was the same whether together or separate. The myometrium releases little PGF but substantial amounts of the weak oxytocic, PGI_2 whilst the deciduum releases substantial PGF with little PGI_2.

The considerable release of AA by decidual PLA_2 in comparison to the myometrium, and its close proximity to the myometrium, could result in the donation of oxytocic PGs to the myometrium. The action of oxytocin , for example, would thus be compounded several fold since it stimulates both decidual PGF and PGI_2, and myometrial PGI_2 synthesis in the pregnant rat (Campos et al., 1982) and both PGs potentiate the action of oxytocin (Williams et al., 1978).

This may be illustrated more clearly in the human pregnant uterus. Ergometrine and oxytocin did not affect human pregnant myometrial PGI_2 synthesis. It has also been shown that oxytocin does not affect PGE or

PGF release by late pregnant myometrium (Fuchs, Husslein & Fuchs, 1981), even though the myometrium and deciduum possess oxytocin receptors (Fuchs, Fuchs, Hesslein & Soloff, 1981). Since the decidua is a substantial source of PG production (as well as the amnion [Schwartz et al., 1975]), PGs must be donated to the myometrium to exert their actions.

The question of the role of the PGI_2 synthesised by the rat and human pregnant myometrium now arises. The differences in the amounts released, action on uterine contractility and the action of oxytocic drugs on the amount released, indicate a difference in the role of PGI_2 between the two species.

The substantial increase in PGI_2 synthesis with the gestational age of the rat creates levels greater than those of PGF, PGE and TxB_2 (Wilson et al., 1982). This increase is probably myometrial in nature with little or no contribution from vascular sources (Williams et al., 1978). The presence of a mechanism for the stimulation and inhibition of synthesis by oxytocic and tocolytic drugs, coupled with the ability of PGI_2 to potentiate the uterotonic activities of PGF and oxytocin certainly suggests a role of PGI_2 in regulating uterine tone of the pregnant rat at term. The lack of such a mechanism and the poor biphasic stimulant and relaxant actions, coupled with comparably low synthetic capacity, suggests that such an action is unimportant in the pregnant human myometrium. However the potent anti-aggregatory and vasoactive properties of PGI_2 cannot be ignored.

An interaction between the proaggregatory substance TxA_2 released by platelets and intimal tissue of vessels, and PGI_2 released by the endothelium is said to exist (Moncada & Vane, 1980). It has already been noted that excessive menstrual blood loss is associated with a high

production of PGI_2 by the endometrium (Smith, Kelly, Abel & Baird, 1981) which could result in vasodilatation and reduced platelet aggregability leading to increased bleeding.

There is an increase in platelet aggregability (Kunze, Briel, Kunze & Schlotter, 1975) as well as TxA_2 release by platelets (Ylikorkala & Viinika, 1980) with the progression of human pregnancy and a reduction in the sensitivity of platelets to PGI_2 in late pregnancy (Briel & Lippert, 1981). Thromboembolic complications are also more common post-partum as opposed to pre- and peri-partum (Beller, 1968), when the myometrium has a reduced capability for PGI_2 synthesis.

Circumstantial evidence that PGI_2 is important in controlling utero-placental haemostasis in the human primarily revolves around observations taken during pre-eclampsia. Pre-eclampsia is characterised by an increase in maternal blood pressure and intravascular coagulation during the third trimester, to such an extent as to threaten the life of the child since utero-placental blood flow is severely depressed. The condition, if left untreated, can develop into eclampsia which is characterised by a further rise in blood pressure, convulsions, abruptio placentae and death. Pre-eclampsia is associated with platelet deposition in the uterine and placental vascular beds (Wardle, 1972). Trophoblastic invasion of spiral and radial arteries is reduced with a concomittant constriction of the radial arteries at the deciduo-myometrial junction (Robertson, Brosens & Dixon, 1976). Trophoblasts synthesise PGI_2 in amounts equivalent to endothelial cells and this could account for the lack of platelet deposition and vascular disease in these vessels which do not possess endothelial cells in late pregnancy. Foetal vessels synthesise greater amounts of PGI_2 than

maternal vessels (Remuzzi, Misiani, Muratore, Marchesi, Livio, Schiepatti, Mecca, de Gaetano & Donati, 1979) which is reduced in preeclampsia (Remuzzi, Marchesi, Zoja, Muratore, Misiani, Rossi, Barbato, Capella, Donati & de Gaetano, 1980). Urinary excretion of 2,3-dinor-6-oxo-$PGF_{1\alpha}$ and 15-oxo-13,14-dihydro-2,3-dinor-6-oxo-$PGF_{1\alpha}$ are also reduced when compared to normotensive pregnant controls (Goodman, Killam, Brash & Branch, 1982). A single case study has reported the platelet counts of a preeclamptic woman and showed a decrease in the maternal platelet count coincident with foetal death (Jespersen, 1980).

In view of these and other reports of reduced PGI_2 synthesis in pre-eclamptic patients it has been suggested that there may be a causal link between the two (Lewis, 1983). Indomethacin certainly reduces uterine blood flow in pregnant sheep (McLaughlin, Brennan & Chez, 1978) and dogs (Terragno et al., 1974). The pregnant dog uterus also synthesises and releases PGI_2 in amounts which could attribute to the control of utero-placental vascular tone (Gerber, Payne, Murphy & Nies, 1981) especially since the placental bed has no neuronal control. Makila, Joupila, Kihonin, Viinika and Ylikorkala (1983) have shown there is a positive correlation between umbilical blood flow and PGI_2 formation in various disease states including pre-eclampsia.

As has already been suggested, placental blood flow could be affected by myometrial PGI_2 release, as well as haemostasis. The anti-aggregatory activity released by the placenta itself though, was not PGI_2 but an ADPase. This ADPase is also released *in vitro* by placental cells in culture (Khoker, Oiller, Dandona, Hutton & Lane, 1981). Although PGI_2 is considered to be a major factor in maintaining haemostasis it must be remembered that donation of ADP by erythrocytes is a contributing factor to platelet aggregation in whole blood and any

release of an ADPase may reduce this cooperation (Born & Kratzer, 1982).

In summary, the experiments undertaken here show that the human and rat myometria synthesise an antiaggregatory AA metabolite, with properties similar to PGI_2. The human placenta was found to release an anti-aggregatory material which could be attributed partly or wholly to a factor with ADP-degrading properties. The capacity of the human myometrium increased over the third trimester to term in a manner similar to the rat, but was found to inhibit spontaneous contractility of the human myometrium.

The capacity of the pregnant rat myometrium to synthesise PGI_2 could be modulated by receptor activation by uterine stimulants and relaxants which may be mediated in turn by myometrial adenylate cyclase activity. Uterine stimulants did not alter the capacity of the human myometrium to synthesise PGI_2.

Phospholipase A_2 activity was described in the pregnant rat uterus which could provide a further basis for research into the action of steroids or drugs on decidual and myometrial phospholipase A_2 activity ex vivo, as well as interrelationships between the deciduum and myometrium.

8.0) Abbreviations

The following abbreviations were used in this thesis:

AA	Arachidonic acid
ADP	Adenosine Diphosphate
ADPase	Adenosine Diphosphatase
cAMP	Cyclic 3',5',- Adenosine mono-phosphate
dPC	Dioleoyl-phosphatidylcholine.
^{3}H-dPC	1'-oleoyl,2'^{3}H-oleoyl-phosphatidylcholine.
5-HT	5-hydroxytryptamine
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
ID_{25}	Dose required to inhibit a response by 25 %.
6-oxo-PGE_1	6-oxo-prostaglandin E_1
6-oxo-$PGF_{1\alpha}$	6-oxo-prostaglandin $F_{1\alpha}$
$PG(letter_{suffix})$	Prostaglandin $(class_{series})$
PLA_1	Phospholipase A_1
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PRP	Platelet-rich plasma
Solution 1	Tris-HCl (100 mMolar)
Solution 2	Tris-HCl (100 mMolar) + Tween 80 (0.1%)
Solution 3	HEPES (50 mMolar) : HEPES (50 mMolar) + 0.2% Triton X100: 0.25 Molar sucrose, (5:3:1.7)
Solvent 1	Hexane:dioxane:glacial acetic acid (70:30:1)
Solvent 2	Chloroform:methanol:water (65:35:4)
Solvent3	Chloroform:methanol:glacial acetic acid:water (50:15:4:2)

9.0) REFERENCES

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